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Acurex Corp, Mountain View, CA

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Terrestrial Ecology Protocols for Environmental Assessment Programs: Workshop Proceedings

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Assessments	Stationary Sources	14B
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Ecology		06F
Bioassay		06A
Plant Physiology		06C
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R.L. Waterland, Compiler

Acurex Corporation
485 Clyde Avenue
Mountain View, California 94042

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EPA Project Officer: Raymond G. Merrill

Industrial Environmental Research Laboratory
Office of Energy, Minerals, and Industry
Research Triangle Park, NC 27711

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ABSTRACT

Through combined efforts of EPA's Industrial Environmental Research Laboratory (IERL-RTP) and Corvallis Environmental Research Laboratory (CERL), a workshop was held in Corvallis, Oregon, during November 1978 to discuss potential tests for inclusion in, and make recommendatons for a terrestrial ecology bioassay testing protocol for use in IERL Environmental Assessment programs. Workshop participants included both government and private researchers in the fields of plant physiology, soil microbiology, and entomology. Specific issues addressed at the workshop included: what tests should be included in a Level 1 protcol, what should Level 1 to Level 2 decision criteria be, and what kinds of tests would be appropriate at Level 2.

This report serves as the proceedings of the workshop. It summarzes key points of discussion and presents the results, conclusions, and recommendations reached in addressing stated workshop issues. Recommended Level 1 plant, soil, and animal assays are discussed, and suggested kinds of Level 2 procedures, based on Level 1 findings, are presented.

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SECTION 1

INTRODUCTION

In 1975 EPA's Industrial Environmental Research Laboratory, Research Triangle Park (IERL-RTP) initiated a series of Environmental Assessment (EA) programs. These programs were designed to:

- Systematically evaluate the physical, chemical, and biological characteristics of all effluent streams from an energy conversion or industrial process
- Predict the probable effects of those streams on the environment
- Rank those streams relative to their individual hazard potential
- Identify and define necessary control technology development programs to reduce the potential hazard presented by those streams

To satisfy these aims, key aspects of an EA are a characterization of the total pollution potential presented by waste streams from a process and a comparison of the nature of the potential environmental insult to existing standards or defined environmental goals.

Types of EA programs currently underway include assessments of low, medium, and high Btu gasification, fluidized bed combustion, stationary conventional combustion, coal cleaning, and coal liquification processes. In performing these EA's, IERL is satisfying one of its roles in supporting the regulatory and enforcement offices of EPA by anticipating future control technology needs and developing the data bases needed to support standards development.

To support the EA programs a tiered source sampling, chemical analysis, and bioassay approach has been defined to provide the data needed to evaluate potential environmental impact. This tiered approach incorporates three levels of sampling and analysis comprehensiveness and detail:

- Level 1: Screening -- structured to identify potential problem effluents and pollutants
- Level 2: Confirmation -- structured to confirm the existence of problem effluents and pollutants

- Level 3: Risk Assessment -- structured to quantify the extent of environmental impact from problem effluents

The tiered approach was adopted because it offered potential cost savings over a direct approach in which all streams would be carefully sampled and completely analyzed in one pass. In the tiered approach effluent streams are first surveyed, or screened, using simplified, generalized sampling and analysis methods (Level 1) which permit ranking streams on a more hazardous to less hazardous basis. Detailed sampling and analysis (Level 2) would then be performed on those priority streams identified at Level 1. Level 2 would thus confirm screening results and provide better quantitative information on potential environmental hazard.

In each level of the tiered approach, both chemical and biological characterizations of an effluent stream are performed. The chemical characterization provides a quantitative, engineering type numerical evaluation of a stream's potential hazard, along with control technology development input. The biological characterization provides a direct measure of a stream's potential hazard in terms of a biological response. In addition, the biological testing aids in identifying toxicant synergisms and antagonisms. Thus the dual chemical and biological characterizations are designed to supplement each other.

To date, through efforts coordinated by the Process Measurements Branch of IERL-RTP, Level 1 sampling and chemical analysis procedures have been defined (References 1, 2), and Level 2 chemical analysis procedures are being defined. However, since IERL expertise is mainly in the fields of chemistry and engineering, a Bioassay Subcommittee of the overall IERL Environmental Assessment Steering Committee was formed to advise and coordinate EPA inhouse and contractor efforts in developing appropriate bioassay testing protocols to parallel the chemical analysis procedures. The subcommittee draws representation from five Office of Health and Ecological Effects laboratories. As such, it represents expertise in the fields of pollutant effects on human health, aquatic ecology, terrestrial ecology, and carcinogenicity and mutagenicity.

Initial subcommittee efforts resulted in defining a preliminary Level 1 bioassay protocol (Reference 3). The prescribed protocol specified testing of whole effluent samples for:

- Mutagenicity (one test)
- Human health effects (two in vitro tests, one in vivo test)
- Aquatic ecology effects (three fresh water tests, three marine tests)
- Terrestrial ecology effects (two tests)

Since little experience with complex effluent testing using any of the tests specified existed, a series of pilot studies were initiated upon publication of this preliminary Level 2 protocol. These pilot studies

were designed in part to validate the protocol and to evaluate the utility of the data obtainable. Results of the pilot studies are still being evaluated. However, based in part on these results and other criticism advanced since publication of the preliminary protocol, concerns have been raised over the propriety of including the terrestrial ecology effects tests specified. Questions concerning ease of sample collection, transport, and analysis, and of ultimate data interpretation have led to concerns over whether other terrestrial ecology tests now being developed might be more appropriate than the ones specified.

In response to these concerns IERL and the Corvallis Environmental Research Laboratory (CERL), organized a workshop to specifically address a recommended terrestrial ecology testing protocol for use in the EA tiered approach. Specific issues to be addressed at this workshop were:

- What should the specific terrestrial ecology test protocols be for Level 1 bioassay testing?
- What should the decision criteria be for specifying Level 2 terrestrial ecology test needs based on Level 1 results?
- What are potential terrestrial ecology protocols for Level 2 bioassay testing?

Specific questions, relating to these issues, needing answers included:

- What specific tests should be used for testing the variety of gaseous, liquid, and solid samples to be encountered?
- What are sample size, integrity, age, etc. requirements for candidate tests?
- Can candidate tests accommodate the currently defined sampling procedures?
- Should Level 2 testing include applying Level 1 test procedures to fractionated effluent samples?
- Are chronic effects tests feasible for Level 2 testing?

The workshop was held on November 14-15, 1978 in Corvallis, Oregon. Technical participants in the workshop are listed in Table 1. The agenda for the workshop included an introductory session, where the background on EA program needs and the ground rules for discussion were established, three working sessions on potential plant, soil, and animal assays respectively, and a summary session.

In the introductory session it was noted that, in developing recommendations for potential test protocols, six criteria were to be considered for candidate tests:

TABLE 1. TERRESTRIAL ECOLOGY BIOASSAY WORKSHOP PARTICIPANTS

Participant	Affiliation
Dr. J. Bromenshenk	Entomologist/Ecologist University of Montana Missoula, Montana
Dr. K. Duke	Associate Manager Ecology and Ecosystem Analysis Section Battelle - Columbus Laboratories Columbus, Ohio
Dr. R. Eagar	Soil Microbiologist Union Carbide Corporation Tarrytown, New York
Dr. J. Gillett Chairman, Soil and Animal Assay Sessions	Research Ecologist U.S. EPA Corvallis Environmental Research Laboratory Corvallis, Oregon
Dr. A. Goloff	Plant Physiologist Union Carbide Corporation Tarrytown, New York
Dr. B. Lighthart	Soil Microbiologist U.S. EPA Corvallis Environmental Research Laboratory Corvallis, Oregon
Dr. D. McCune	Plant Physiologist Boyce Thompson Institute for Plant Research Ithica, New York
Dr. R. Merrill Workshop Chairman	Research Chemist U.S. EPA Industrial Environmental Research Laboratory Research Triangle Park, N.C.
Dr. R. Rogers	Soil Microbiologist University of Nevada Las Vegas, Nevada

TABLE 1. Concluded

Participant	Affiliation
Dr. W. Rosen	Plant Physiologist U.S. EPA Office of Toxic Substances Washington, D.C.
Dr. S. Sandhu	Research Biologist U.S. EPA Health Effects Research Laboratory Research Triangle Park, N.C.
Dr. D. Shriner	Research Ecologist Oak Ridge National Laboratory Oak Ridge, Tennessee
Dr. T. Tibbitts	Professor of Horticulture University of Wisconsin Madison, Wisconsin
Dr. D. Tingey Chairman, Plant Assay Session	Plant Physiologist U.S. EPA Corvallis Environmental Research Laboratory Corvallis, Oregon
Dr. L. Waterland Meeting Coordinator	Leader, Process Analysis Section Acurex Corporation Mountain View, California

- Cost
- Sample requirements (form and quantity)
- Relevance to terrestrial ecology and biology
- Availability (existence of accepted test procedures and a validation data base)
- Comparability (among different laboratories performing the test)
- Response (easily measured and sensitive)

Pertaining to these criteria it was noted that, for Level 1, tests must cost no more than $500 to $700 and must take no more than 2 to 3 months to complete from receipt of samples to test report completion. In addition, it was noted that, based on current Level 1 sampling procedures, available sample quantities for liquid and solid effluent streams would be essentially unlimited (within reason), but that gas stream particulate and organic species (sorbent extract) samples would probably be limited to 250 to 300 mg quantities. Based on these constraints, discussion in each of the working sessions focused on the above issues and criteria.

This report serves as the proceedings of the workshop and presents the results, conclusions, and recommendations reached in defining a Level 1 terrestrial ecology bioassay protocol, suggesting potential Level 2 protocols, and outlining decision criteria for specifying Level 2 tests based on Level 1 results. As such, the remainder of the report is organized as follows. Section 2 presents a summary of general discussion and overall concerns raised at the workshop which do not specifically relate to a given (plant, soil, animal) session topic area. Sections 3 through 5 summarize discussion specific to each topic area: plant, soil, and animal assays respectively. Section 6 summarizes overall workshop conclusions and recommendations. Finally, several of the technical participants listed in Table 1 served as EPA consultants. Their reports on conclusions and recommendations reached are reproduced in the Appendices.

SECTION 2

GENERAL DISCUSSION

Throughout the workshop, even into the summary sessions, several issues or concerns of a general nature were raised, issues not strictly focused on the discussion of a given class (plant, soil, animal) of candidate assays for incorporation into the EA phased analysis approach. This general discussion is summarized in this section.

2.1 DEVELOPING STATE OF TERRESTRIAL ECOLOGY

The first of these general points of discussion concerned the current relatively undeveloped state of the terrestrial bioassays. Specifically it was noted that research in the field of toxicant effects on terrestrial ecology lags that in the areas of carcinogenesis/mutagenesis, human health effects, and aquatic ecology effects by several years. Compounding this is the fact that, although several government groups want to employ terrestrial bioassay testing, few are funding developmental work and coordination of ongoing efforts is poor. Thus, virtually all existent terrestrial bioassay test procedures must be considered developmental. Few terrestrial assays have been validated, and experimentation with other than pure compounds has not been performed. Of course it should be noted that experience with complex effluents testing was largely lacking for all tests proposed in the preliminary Level 1 bioassay protocol prior to initiating the EA pilot studies. But this fact must be underscored for terrestrial ecology tests, and supplemented by noting that the degree of pure compound testing in terrestrial ecology protocols has been limited. Therefore, it must be emphasized that any set of test procedures recommended now for inclusion in the EA methodology should be taken only as the best available at present, given the constraints that a test must be inexpensive, simple, and reliable. However, as other test procedures develop, and interpretation of test data becomes more discriminating, other protocols may become more appropriate.

2.2 FEATURES OF APPROPRIATE TESTS

The above led into the next general area of discussion: what generic kinds of tests would be appropriate for incorporation into the tiered approach? For example, is it necessary for a test just to give a response when subjected to an effluent sample, or should we expect the response to be indicative of some real environmental impact, such as crop loss? This kind of question relates closely to the above discussed state of development of terrestrial bioassays. Several test procedures are

currently being developed which give responses other than lethality to known toxicants. However, quantitatively relating these responses to effects on an organism's growth, development, and reproduction is often quite difficult. Furthermore, the ties between these specific effects on an organism and more global impacts on an ecosystem are even more tenuous.

With this in mind, it was decided to focus attention on tests that have broad applicability and integrate organism responses. Responses measured should, where possible, be correlatable to effects on the test organism's growth, development, and/or reproduction. In addition, recommended protocols should include, as a minimum, a representative test for each of the producer, consumer, and decomposer (recycler) organism classes. In this regard, though, discussion should focus on bioassays that measure toxicity and not mutagenicity.

Another concern in this area related to the question of the what are acceptable levels of false positive and false negative responses in a given test. Clearly, what is desired in any candidate test is a minimum of both. However, this is not generally possible. Thus, it was decided that for Level 1 screening tests, a minimum of false negative responses was key, with a reasonable level of false positive responses acceptable. Level 2 testing would be designed to remove the false positives. Of course, it must be noted here that, for most terrestrial ecology assays, little is currently known about the incidence of false positive or negative responses. Thus, this information must await future test development and validation.

Finally, discussion focused on the eventual need to include chronic effects testing. Here, the needs of an EA program, and the desire to identify chronic toxic effects are somewhat at odds. An EA requires rapid test procedures, especially at the Level 1 stage, but at Level 2 as well. But chronic effects testing, by its very nature, requires lengthy test times, which currently fit well only in Level 3. There is concern, though, that by delaying chronic effects testing to Level 3, many potential effluent streams would have been screened out at Levels 1 and 2. In fact, IERL has requested that the bioassay subcommittee consider specifying a chronic effects or life cycle test for optional use on Level 1 samples. Though this would be considered a Level 2 test, testing of Level 1 samples would be considered when indicated. What is required is an inexpensive, simple, short term chronic effects test. Although, none is currently available, such a test may become available in the future. For example, the Arabidopsis bioassay being developed at the Corvallis Environmental Research Laboratory is a short term life cycle test which satisfies some of the needs of a chronic effects test. Thus it was decided that, of necessity, chronic effects testing may not be addressed at Level 1, but some form of life cycle testing should be incorporated into the Level 2 protocol when it becomes definitely established. Hopefully, some life cycle tests, such as the Arabidopsis bioassay, will be well developed in time for inclusion into a detailed Level 2 protocol.

2.3 DIFFICULTIES OF GAS SAMPLE TESTING

The next general area of discussion concerned the difficulty of gas sample testing. Although gas fumigation techniques are standard procedures and can be adapted to virtually all terrestrial assays, most current bioassay procedures require very large volumes of sample. This problem is compounded if flow through testing is required instead of static gas sample testing. In this regard, flow through testing is the preferred approach since it is often difficult to make a valid assessment of dose-response under static exposure conditions.

Large gas samples are difficult to collect in the field, transport to the laboratory, and store until use. Compounding this problem is the fact that collecting large gas samples requires using some form of plastic (e.g., Tedlar) bag. Sample component loss through the bag, chemical reactions with the bag material, and absorption to the bag walls have been experienced (Reference 4), leading to a very real concern about sample integrity by the time it is assayed.

These problems can be circumvented by performing tests onsite in the field. But this approach introduces a different set of problems. Besides it would be inordinately expensive, and not feasible at some test sites. Gas sample compression or cooling would reduce the volume requirement for sample transportation and storage, but this approach also suffers from maintenance of sample integrity questions.

The best solution to the gas sample testing problem would involve decreasing the sample size requirement of a given test procedure; in other words, miniaturizing the test. However, this must be considered more of a long term solution. For example, work is currently proceeding toward miniaturizing the stress ethylene protocol, but results are not expected for at least 24 months. Therefore, it was decided that, for the present, gas stream testing be performed in the laboratory, using static procedures to minimize, to the extent possible, sample size requirements, and hope that the sample tested remained sufficiently representative of the gas stream sampled to give trustworthy results. In this respect, it was noted that, although some sample loss and chemical reactions might occur, a gas sample would probably maintain its phytotoxicity for some reasonable time period. Furthermore, given the screening nature of Level 1, the loss of some component or some chemical transformation might not seriously invalidate test conclusions at Level 1. Moreover, if Level 1 tests were positive on these potentially degraded gas samples, one might conclude that the sample contained highly toxic and stable compounds. For the future, though, the development of "miniature" test assays should be strongly encouraged, and the use of flow through gas sample testing pursued over static testing.

2.4 DECISION CRITERIA

The propriety of specifying Level 1 to Level 2 decision criteria as a workshop output was also raised as an issue. After discussing the most recently proposed decision criteria for the other (mutagenesis, health effects, aquatic effects) tests in the Level 1 protocol, and noting the

tentative nature of these criteria, it was decided to leave specifying detailed criteria to future Bioassay Subcommittee efforts. This was deemed appropriate since the subcommittee is in the best position to assure consistent decision criteria among all mutagenesis, health effects, aquatic, and terrestrial tests. However, the workshop would offer guidance as to what kinds of Level 2 testing would be indicated based on Level 1 results.

In general, it was agreed that:

- Level 2 terrestrial ecology testing emphasize using Level 1 test procedures on effluent sample fractions if terrestrial ecology tests give positive, or high toxicity responses

- Level 2 terrestrial ecology testing emphasize testing unfractionated samples with other species and other responses, and life cycle testing if Level 1 terrestrial ecology tests give negative, or low toxicity responses, but other Level 1 tests (health effects, aquatic effects) give positive, or high toxicity responses.

These recommendations are summarized in more detail in the test assay sections (Sections 3 to 5) of this report. It should be noted that these recommendations conform to the objectives of Level 2: to confirm Level 1 results and to isolate toxic species through fractionation. High toxicity streams should not need confirmation and could go directly to fractionation. Lower toxicity streams should need confirmation.

Tempering the above recommendation was the observation that the most care in describing Level 1 to Level 2 decision criteria is required in cases where samples are of moderate toxicity. Level 2 would definitely be indicated for samples which gave high toxic responses in all biotests and triggered high in the Level 1 chemical analyses. Conversely, Level 2 testing would be of low priority for samples which elicited no response in any bioassay and triggered low in the chemical analyses. It is in the middle ground, where samples elicit low to moderate toxicity in several tests, or the range of responses varies from not detectable to high toxicity over several tests, that the most difficulty in making a Level 2 decision will be encountered. One possible approach might include the following:

- A high toxicity response in any test should automatically raise a stream's priority

- A moderate toxicity reponse both in one health test and one ecological test should raise a stream's priority

- A low toxicity response in one health test, one aquatic ecology test, and one terrestrial ecology test should raise a stream's priority

With respect to reporting Level 1 results, the method of assigning relative toxicity values (not detectable, low toxicity, moderate toxicity,

high toxicity) based on the maximum applicable dose in a test and the LD_{50}, LC_{50}, or EC_{50} response of a test, as reported in Reference 5, was deemed appropriate.

In a related vein, it was noted that some form of pre-Level 1 decision criteria should also be specified, based on a stream's chemical characterization. For example, a stream with very low pH, or with significant quantities of arsenic present would probably not need to be subjected to bioassay.

2.5 QUALITY ASSURANCE

The next area of general discussion revolved around specifying quality control and quality assurance procedures. In other words, what must be done to assure that good data giving interpretable results come from a given procedure. Three general areas must be addressed here:

- The mechanics of sample and data handling including sample chain of custody records and prevention of data transcription errors
- Individual test procedure quality assurance including provisions for negative control samples (blanks), positive control samples, and audit samples.
- Test results reproducibility and interpretation.

It was agreed that the burden for several aspects of specifying a QA procedure should logically fall to IERL and the Bioassay Subcommittee. Specifically, outlining chain of custody and data transcription checking procedures and providing laboratory audit samples fall in this category. However, specifying positive control compounds will be the responsibility of the individual drafting the detailed procedures document for each test. Thus, appropriate positive control species are discussed below in each test assay section of this report. In addition, it was decided that to ensure test validity and reproducibility, each effluent tested should be assayed at a minimum of three concentration levels, in addition to controls, and that four replicate tests be performed.

2.6 INTRA-EPA PROTOCOL COMPARABILITY

The final general area of concern related to assuring that the bioassay protocol specified for use in IERL EA programs be comparable, where possible, to protocols being developed and employed by other EPA organizations. Currently, due to mandates under the Clean Air Act Amendments, the Clean Water Act Amendments, the Toxic Substances Control Act (TOSCA), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and the Resource Conservation and Recovery Act (RCRA), several EPA offices including the Office of Toxic Substances (OTS), the Office of Solid Waste (OSW), the Office of Pesticide Programs (OPP), and the Office of Water Planning and Standards (OWPS) are developing bioassay protocols to assess the impact of various process, product, and waste streams. These are in addition to the subject IERL protocols. Thus striving for

comparability, to the degree possible, among these protocols, adopting standard procedures, standard controls, standard data interpretation procedures, etc., would seem to be quite important. Of course the needs of an EA program may not quite mesh with those of other offices, but where possible a consistent set of assays should be adopted.

It was noted that IERL is indeed attempting to perform this activity. A representative from OTS was a workshop participant, in part to relate recent OTS efforts in developing protocols for use in TOSCA mandated assays. A document summarizing the specific procedures recommended for use in implementing TOSCA is scheduled for release in early 1979. Findings of this study, and others like it, should be incorporated as appropriate into the IERL protocol development.

SECTION 3

PLANT BIOASSAYS

This section summarizes workshop discussion focused on identifying, weighing, and selecting plant test procedures for inclusion in Level 1 and Level 2 bioassay protocols. As noted in Section 2.2, it was generally agreed that effects on photosynthetic systems (producers) needed to be addressed in the protocol, and that the current aquatic algal tests were not sufficient. Thus, defining specific terrestrial plant tests for use with solid, liquid, and gaseous samples was addressed in this session.

3.1 CANDIDATE TESTS

The discussion opened by listing candidate tests for consideration without regard to applicability (solid, liquid, or gas samples). Tests suggested were:

- Stress ethylene
- Foliar injury
- Seed germination
- Seedling growth
- *Tradescantia* (mutagenesis)
- Pollen viability
- *Arabidopsis* life cycle
- Pea tendril coiling
- Tomato petiole angle
- Cucumber leaf enlargement
- Bean hook opening
- Turgor changes

However, preliminary discussion quickly removed from consideration the last five in the list as being too undeveloped with respect to other tests to be considered at present.

The stress ethylene test is currently included in the preliminary Level 1 protocol for gas sample testing (in fact it is the only test presently specified for whole gas sample testing). However, the test can be used to assay liquid and liquid extracts of solid samples as well (administration by foliar spray or root irrigation). It is one of the more developed of the plant tests, being extensively used by the Corvallis Environmental Research Laboratory (CERL) among others. The test gives a general, integrated, reproducible response. (CERL results have been corroborated by the Battelle-Columbus Laboratories). It is a quite sensitive test; no false negative or false positive responses have been detected. In addition, increased ethylene production has been correlated with decreased growth, establishing the desired link between test response and physiological function. The test cost is about $800 per test series (blank, positive control, three sample concentrations, four replicates). A static gas fumigation procedure has been developed, though a large sample quantity (1300 ℓ) is still required. To overcome this drawback, work to miniaturize the procedure is currently underway, though the miniature procedure will not be available for at least 24 months.

The foliar injury test is also relatively well developed and can be used to assay extracts of solid, liquid, and gas samples through fumigation, foliar spray, or root irrigation. It is a less sensitive test than the stress ethylene procedure, though it has been shown to be dose responsive and reliable. The potential for more false negative responses exists than with the stress ethylene test, however, the incidence of false positive responses should be low.

The seed germination and root elongation assays can probably be considered the most developed assays for liquid samples, though extracts of solids and possibly solids can be tested. Well documented procedures for performing these tests exist (References 6, 7). The root elongation test is attractive because it provides an evaluation of morphological development of plant systems. It is a more sensitive measure of effects than seed germination, which is actually a measure of lethal effect, though both tests are attractive for EA needs. A variety of seeds can be used to detect species response variations.

The Tradescantia assay is a quite sensitive mutagenesis test. However, SO_2 is highly toxic to the test organism, thus the presence of SO_2 in the test sample can mask any mutagenic response. For this reason (SO_2 will almost always be encountered in gas samples from an energy conversion process) and the fact that the test is only in the early stages of development, it is not as attractive an alternative for current EA needs. Moreover, since the workshop focused on identifying candidate tests to measure toxicity, not mutagenicity, the Tradescantia assay seemed inappropriate.

Similarly for the pollen viability assay. In spite of the fact that this test would require small samples for gas testing and is quite

sensitive to SO_2 and inorganic compounds, it hasn't been tested widely and is therefore not sufficiently developed for current needs.

The Arabidopsis test offers the potential for being a rapid, simple, inexpensive life cycle test. But it also is too undeveloped to be used at present.

3.2 RECOMMENDED LEVEL 1 PROTOCOL

Based on the above discussion, the recommended plant assays for incorporation into the Level 1 protocol are:

- The stress ethylene test coupled with the foliar injury assay
- The root elongation test coupled with the seed germination assay

The stress ethylene/foliar injury test is proposed for use with gas samples, with optional use on liquid, solid leachate, and possibly solid samples. The seed germination/root elongation assay is proposed for use on liquid and solid extract samples.

The combination stress ethylene/foliar injury procedure was chosen because it provides the sensitivity of the stress ethylene test with a measure of response severity provided by the foliar injury test. Although the stress ethylene protocol is less well developed for use with liquid and solid extract samples, enough experience with it exists to justify suggesting it for these samples. A static fumigation procedure was recommended for gas sample testing and root irrigation for liquid samples and extracts of solid samples. Some concern was expressed over the organic content of the plant growth medium buffering the toxicity of samples tested. However, the concensus opinion was that this can be avoided with care and proper medium choice. Use of a low organic medium was suggested.

The combination seed germination/root elongation test was proposed for liquid and solid extract sample testing. Incorporation into the germination/growth medium was proposed for liquid and solid leachate sample testing. The choice of species to be used was deferred until actual protocol drafting. Current OTS thought suggests using six species.

Positive control compounds suggested for both tests were ozone or chlorine for gas samples, and some choice of compounds from the reference list of 10 being used by CERL in stress ethylene test work for liquid and solid extract tests (Reference 7). The need to rigorously define plant culture conditions in eventual protocol drafting was emphasized.

The concern that neither of the suggested procedures does life cycle testing was expressed. But, as noted in Section 2.2, it was decided to defer life cycle testing to Level 2, or until the Arabidopsis assay becomes sufficiently developed.

3.3 SUGGESTED LEVEL 2 PROCEDURES

Suggestions for appropriate kinds of tests for Level 2 included:

- Testing for the same (Level 1) responses with other species
- Testing for other responses with the same (Level 1) species, testing responses indicative of effects on a different stage of life, or full life cycle testing
- Testing fractionated effluent samples using the Level 1 test procedures.

Which of these kinds of tests would be called for would depend on Level 1 results obtained. If the Level 1 plant tests gave positive (toxic) responses, then Level 2 would emphasize sample fraction testing using the same Level 1 procedures. If the Level 1 plant tests gave negative or low toxicity results, but other bioassay tests triggered positive, then Level 2 plant testing would emphasize testing with other species, and testing other responses, or other life cycle stages with Level 1 species. It was recommended that both sample fractionation and effect confirmation were important facets of Level 2, though the focus on one of these should be determined by what was found at Level 1.

SECTION 4

SOIL ASSAYS

As noted in Section 2.2, a responsive bioassay protocol for terrestrial ecology effects should include representative tests for effects on each of producer, consumer, and decomposer organisms. Plant assays discussed in Section 3 focused on producers; soil assays discussed in this section emphasize decomposers.

4.1 CANDIDATE TESTS

As in the plant session, the soil assay session opened by listing candidate test procedures for consideration. Those advanced were:

- Soil core microcosm
- Endogenous respiration
- Specific microbial tests in culture, or in homogenized soil, measuring:
  - -- Specific respiration/substrate degradation
    - Starch
    - Cellulose
    - Pectin
    - Protein
  - -- Nitrogen fixation
  - -- Nitrification
  - -- Sulfate reduction
  - -- Hydrogen (tritium) oxidation
- Sludge testing

Sludge testing was rather quickly eliminated from further consideration because these are somewhat poorly defined tests with no standard

procedures established. Furthermore interpreting data from these tests is relatively unclear.

The use of cultured bacterial colony testing was also rejected. Interesting organisms tend to be difficult to culture and single strain testing lacks the broad sensitivity and integrated response aspects desired of a Level 1 test.

Soil testing provides the desired integrated responses. However, it must be remembered that soil testing can mask potential effects; soil samples can be resistant to many toxic compounds and soil can rebound from an initial insult. Thus cultured organisms can be highly sensitive to toxic effects, whereas soil cultures would show less sensitivity. However, this may not be a detriment; such behavior is more indicative of real world impacts. Besides, the rebounding characteristics of soil colonies allows differentiating between reversible and irreversible effects.

The soil core microcosm test is currently specified in the preliminary Level 1 protocol. However, its use as a Level 1 screen is probably not warranted. The test is quite information-rich, but interpretation of all the data provided is not a straightforward task at present. Thus, as it stands the test can be considered too sophisticated for use in Level 1. Besides, the test is too expensive ($1300 to 2500) and too lengthy (8 weeks) for Level 1 needs.

Many of the objections to using the microcosm test would be overcome if only a single response was measured. However, the choice of an appropriate response is not simple. Calcium efflux (specified in the current Level 1 protocol) is the easiest to measure and has a very low coefficient of variation. Phosphate, sulfate, and nitrogen (ammonia, nitrite, or nitrate) export are also potential responses. However, these are all species likely to be found in text effluent samples, so test results are liable to be difficult to interpret. Carbon dioxide release (also specified in the current Level 1 protocol) as a measure of respiration is also a possibility, but again, data interpretation would be difficult. As testimony to the above, what little work has been done with the soil microcosm test on complex effluents is seemingly contradictory and difficult to interpret.

It was agreed, then, that use of the soil core microcosm test not be recommended for use in a Level 1 protocol, but perhaps be further developed for use in Level 2 assays. For current Level 1 needs it was agreed that homogenized soil testing offered the best approach. Thus, the choice of appropriate response to measure remained. Both specific respiration and hydrogen oxidation were deemed too undeveloped at present. Hydrogen oxidation as measured by tritium conversion to water may prove, in the future, to be a very rapid, inexpensive, simple response, perfect for Level 1 needs. However, current experience at the University of Nevada, Las Vegas, and the EPA Environmental Monitoring and Support Laboratory, Las Vegas, has only been developed with SO_2, cadmium, and mercury.

The most promising of the remaining responses were concluded to be endogenous respiration (as measured by CO_2 release), nitrogen fixation (as measured by acetylene reduction), and nitrification.

Endogenous respiration is a stable function, though not particularly sensitive. However, much experience with measuring this response exists. Work at CERL has shown a very low coefficient of variation can be obtained (1.6 percent) in the rate of CO_2 released versus time after the initial rapid release with test sample addition has occurred. However, CO_2 must be monitored for at least 30 days to allow soil reequilibration, before irreversible toxic effects can be identified.

Both nitrification and nitrogen fixation are quite sensitive functions. But nitrogen fixation is perhaps the more developed of the two tests.

4.2 RECOMMENDED LEVEL 1 PROTOCOL

Based on the above discussion, the recommended soil assay for incorporation into the Level 1 protocol is a homogenized soil test, where endogenous respiration (CO_2 release), and nitrogen fixation (acetylene reduction) are monitored. These two functions represent a combination of a stable, though less sensitive, response (endogenous respiration), and a highly sensitive response (nitrogen fixation), capable of identifying irreversible toxic effects

Table 2 shows required test time, estimated test costs (for blank, positive control, and three sample concentrations testing, four replicate tests), and potential positive controls for each response. The test time requirement is in addition to the initial 14 days equilibration period before test sample addition. It was agreed that 30-day monitoring of CO_2 release was needed to allow the mitigating properties of the soil to take effect. A shorter, perhaps 14 day, test could be specified, but this would also capture reversible effects and the rebounding properties of soil would not be observed. Of course it could be argued that assaying for immediate responses is appropriate for Level 1 screening, with system resiliancy being an appropriate response to look for at Level 2. But, it was noted that if the endogenous respiration assay was limited to short term effects, the incidence of false positive responses might be unacceptably high and much of the screening nature of the test might be lost. Thus the longer term endogenous respiration test was recommended.

The recommended soil assay was proposed for use on all solid, liquid, and gaseous samples. Liquid samples and leachate testing would be introduced by soil irrigation and solid samples by mixing with the test soil. Static gas sample testing would be performed by placing a test atmosphere over the soil sample. Although there has been very little experience with testing gas samples using this technique, it was deemed straightforward enough to warrant inclusion.

In actual test implementation it would be wise to attempt to define a standard soil to be used for all testing. A soil of a certain type, obtained from a well-defined geographical location would suffice. A

TABLE 2. RECOMMENDED LEVEL 1 SOIL ASSAYS

Test Response	Time Required	Estimated Cost	Candidate Positive Control Compounds
Endogenous Respiration	30 days	$500-1000	Gas: Ethylene oxide Liquid: 2,4 dinitrophenol, Solid: Silver or cadmium compound
Nitrogen Fixation (C_2H_2 reduction)	24 hours	$500-1000	Gas: Ethylene oxide Liquid: Sodium azide Solid: Silver or cadmium compound

synthetic soil would meet the standard soil needs, but work to develop a good synthetic soil has been singularly unsuccessful. Homogenization procedures should also be standardized. Final soil particle size should be less than 2 mm.

For the future it was emphasized that other responses may become more appropriate for Level 1 needs as they become developed. Hydrogen oxidation and nitrification seem particularly promising in this respect. In fact, hydrogen oxidation shows such good promise that its active development deserves encouragement. It was suggested that pilot testing, not only of the suggested Level 1 tests, but also the hydrogen oxidation and nitrification assay procedures be considered.

4.3 SUGGESTED LEVEL 2 PROCEDURES

Analogous to what was suggested for appropriate Level 2 tests in the plant assay discussion, the kinds of soil assays suggested for consideration at Level 2 included testing effluent fractions using the Level 1 procedures, and testing whole effluents using different tests or monitoring different responses. Specifically, the soil core microcosm test, and homogenized soil assays measuring specific respiration of a variety of substrates were proposed. The use of a nitrification assay as a backup was also suggested.

Again, as for plant assays, the emphasis given to fractionation or confirmation in Level 2 would be based on what was found at Level 1. If the Level 1 soil assay tests indicated significant toxicity, then effluent fraction testing would be emphasized. However, if the Level 1 soil assay tests gave negative responses, but other tests in the Level 1 protocol triggered positive, then soil core microcosm and specific respiration testing would be emphasized.

SECTION 5

ANIMAL ASSAYS

With plant assays satisfying the need to test producer organisms and soil assays satisfying decomposer needs, the third workshop session treated animal assays which focus on consumer organisms. The need for fast, simple assays which are inexpensive, require minimum test organism maintenance costs, and which require small sample size, caused discussion to focus immediately to insect assays. In addition, a higher animal test using rats is already specified in the Level 1 health effects protocol. Thus, only insect assays are discussed below.

5.1 CANDIDATE TESTS

As in the plant and soil sessions, discussion in the animal assay session opened by listing candidate test procedures. Candidate test species suggested were honeybees (Apis mellifera), fruit flies (Drosophila melanogaster), houseflies, and mosquito larvae, as much single compound assay work has been done with all these species in the past. Appropriate responses to measure included lethality (acute LC_{50}), bioaccumulation, enzyme activity, and behavioral alteration. However, it was quickly decided that, for Level 1 needs, lethality was the simplest response to measure, though the life span shortening endpoint deserves some consideration (test time required increases to 3 weeks). Assaying gaseous (fumigation), liquid, and solid samples (ingestion) using these insects would be straightforward.

Honeybee testing is widely used, with standardized procedures being employed by many laboratories throughout the country. Honeybees have the advantage of possessing a clean genetic line; all bees in a colony are derived from a single queen which lives an average of 8 years. Thus bees don't easily develop resistance to toxic compounds. In addition, honeybees are often more sensitive organisms than Drosophila. Using bees as a test organism would also allow a logical sequence of test procedures to be defined through Level 2 to Level 3, where community and social studies could be performed. An auxillary advantage bees have over other insects is the fact that they are a beneficial insect, commercially important as honey producers and pollenators.

Drosophila testing is also quite widely used, thus this insect, and toxicant effects on it, are also well understood. Drosophila colonies are also easier to manage in a laboratory setting, and Drosophila are smaller organisms than honeybees, thus a smaller sample would be required for

assay needs. Houseflies and mosquito larvae have no clear advantages over Drosophila as test insects. The fact that they are pests tends to act subtly to their disadvantage.

5.2 RECOMMENDED LEVEL 1 PROTOCOL

Based on the above discussion the recommended animal assays for use in Level 1 testing are the use of honeybees and Drosophila to assay gaseous (fumigation), liquid, and solid (ingestion) samples. Acute ("inhalation", or oral) LC_{50} was the suggested biological response. Estimated assay costs are in the $300 to $500 range. Caged insect assays were proposed. The use of methyl parathione, Sevin^{R} or other carbamate insecticides, or monosodium methane arsenate as positive control compounds was suggested.

Specific procedures will await detailed protocol drafting. To aid in this, the results of a recent workshop organized by EPA's Office of Pesticide Programs (Dr. A. Vaughan, Coordinator) will become available in the near future. The workshop, held in Washington, D.C. on November 8-9, 1978, and entitled "Conference to Develop Test Methods for Determining Pesticide Effects on Bees," was organized to specifically draft procedures for short term caged assays as well as chronic, 60-day, outdoor tests. Results from this workshop should be available in early 1979.

5.3 SUGGESTED LEVEL 2 PROCEDURES

As with the plant and soil assays discussed above, suggested Level 2 animal tests will include effluent fraction testing and whole effluent testing using different species (houseflies and mosquito larvae were proposed) and measuring different responses (bioaccumulation, behavior modification, and enzyme activity were recommended). Emphasis on fractionation or confirmation testing will be applied based on Level 1 results, as discussed in Sections 3.3 and 4.3. The potential for including a Drosophila mutagenicity assay at Level 2 also exists.

SECTION 6

SUMMARY AND CONCLUSIONS

The recommended terrestrial ecology tests for incorporation into the Level 1 bioassay protocol are listed in Table 3, which also indicates the suggested method of sample administration for each potential sample type (gas, liquid, solid). Specifying the specific details of each individual procedure is deferred until actual protocols are drafted. The responsibility for drafting individual protocols will be assigned by the Bioassay Subcommittee of the IERL Environmental Assessment Steering Committee.

The fact that none of the tests recommended for Level 1 can really be considered adequately developed deserves emphasis. For all the tests, experience with complex effluent testing is nonexistent, and for several of the tests pure compound experience is limited. In addition, experience with gas sample testing using the soil assay procedures, with liquid and solid sample testing using the stress ethylene test, and with solid (not leachate) sample testing using all the plant and soil tests is deficient. For these reasons, pilot testing of all the proposed procedures is strongly recommended before they become included in a definite Level 1 protocol. In addition, it must be emphasized that any set of test procedures recommended now for inclusion in the EA methodology should be taken only as the best available at present, given the constraints that a test must be inexpensive, simple, and reliable. As other test procedures develop, and interpretation of test data becomes more discriminating, other protocols may become more appropriate.

Suggested kinds of procedures for Level 2 tests include:

- Effluent sample fraction testing using Level 1 procedures
- Testing for Level 1 responses in other test species (e.g., houseflies and mosquito larvae in the animal assays)
- Testing for different responses or testing for effects on different life cycle stages with Level 1 tests species (e.g., specific respiration assays with a variety of substrates in the soil assay; bioaccumulation, enzyme activity, and behavior modification in the insect assays)

In addition the soil core microcosm test is recommended as a Level 2 soil assay.

TABLE 3. RECOMMENDED LEVEL 1 BIOASSAY PROTOCOL FOR TERRESTRIAL ECOLOGY

Plant Tests	Soil Tests	Animal Tests
1. Stress ethylene and foliar injury: • Gas: Fumigation • Liquid: Root irrigation (optional) • Solid: Medium incorporation (optional)	1. Homogenized soil assay measuring: -- Endogenous respiration (CO_2 release) -- Nitrogen fixation (C_2H_2 reduction)	1. Honeybee acute LC_{50}: • Gas: Fumigation • Liquid: Ingestion • Solid and Solid Leachate: Ingestion
2. Seed germination and root elongation • Liquid: Irrigation • Solid: Leachate irrigation	• Gas: Fumigation • Liquid: Irrigation • Solid: Soil incorporation and leachate irrigation	2. Drosophila acute LC_{50} • Gas: Fumigation • Liquid: Ingestion • Solid and Solid Leachate: Ingestion

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Specific criteria for deciding Level 2 test needs based on Level 1 results should be outlined by the Bioassay Subcommittee as a whole. However, it is proposed that:

- Level 2 terrestrial ecology testing emphasize using Level 1 test procedures on effluent sample fractions if terrestrial ecology tests give positive, or high toxicity responses

- Level 2 terrestrial ecology testing emphasize testing unfractionated samples with other species and other responses if Level 1 terrestrial ecology tests give negative, or low toxicity responses, but other Level 1 tests (health effects, aquatic effects) give positive or high toxicity responses

In addition to the above protocol recommendations, several other points deserve emphasis. These include:

- The development of other test procedures and other test responses for potential inclusion into the phased analysis should be encouraged. Specific tests/test responses showing promise for meeting Level 1-2 needs include:

  -- The Tradescantia assay

  -- Miniaturization of the stress ethylene procedure

  -- The Arabidopsis assay as a potential life cycle test

  -- Monitoring nitrification in a soil assay

  -- Monitoring hydrogen oxidation in a soil assay

- A rapid simple chronic effects assay is sorely needed. The Arabidopsis procedure shows some promise here

- The need to ensure comparability between the IERL protocol and those being defined by other EPA offices is of critical importance

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APPENDICES

CONSULTANTS' REPORTS

APPENDIX A

D. Tingey
EPA/IERL

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
Corvallis Environmental Research Laboratory

SUBJECT: Bioassay Workshop held in Corvallis November 14 & 15 DATE: November 27, 1978

FROM: David T. Tingey

TO: File

The bioassay workshop meeting opened with a discussion presented by Dr. Ray Merrill from IERL, RTP, who presented an overview of the environmental assessment program and the role of the bioassays in the program, sample constraints, and sample limitations. Also he defined level 1 bioassays as screening bioassays and level 2 bioassays as confirmatory bioassays.

Specific discussions were held to select the bioassays for plants, soils, and animals, with the constraints that the bioassays selected must be already developed but not necessarily validated. Also there was no money available for any new level 1 bioassay development.

Level 1 bioassays selected for plants were: Phytotoxicity to include stress ethylene and foliar injury, and a test on plant growth probably root elongation similar to the test we are developing for OTS. The soil bioassays would incude a time for the soil to equilibrate and then it would be stressed and for short term changes in respiration, and in a specific process, such as nitrogen fixiation would be measured. As a level 1 bioassay, there was also expressed interest in the tritium oxidation system developed by Las Vegas, but there was no money available to develop the protocol. Level 1 bioassays proposed for the animal system were to determine LC_{50} for honeybee and for drosophila.

Level 2 bioassays for terrestrial systems would be initated under either of two criteria. One the terrestrial test level 1 bioassays showed high toxicity. If this were the case then Level 2 bioassays would be run of fractioned samples using the same tests as Level 1. However, if level 1 bioassays for the terrestrial component did not show high toxicity but either the aquatic or the human health tests show high toxicity, level 2 bioassays would be run on unfractioned samples and would include additional species in the same tests.

The specific level 2 protocols for plants would include phytotoxicity tests, a root elongation test as proposed in level 1 and would also include a full life cycle test, most likely Arabidopsis. Level 2 soil tests would include a soil microcosm and specific substrate respiration using C-14 labeled compounds such as starch, pectin, cellulose, and protein. Level 2 animal bioassays would include behavioral changes in addition to LC_{50} data.

The two proposed grants to be funded in FY 79 from the IERL money were discussed. The proposal of Lyle Craker to miniaturize the stress ethylene system was well received by the workshop participants. They felt this was a valuable addition to the bioassay protocol systems. However, the grant proposal of Ken Williamson from OSU did not receive a good reception from the woshop participants.

Protocols have been proposed for plants soils and animals for both level 1 and level 2 as a result of the workshop. It is now necessary to determine if all of the proposed protocols will be implemented in a Level I sampling program. For the tests selected at levels 1 and 2 protocols will need to be prepared and these protocols will need to be validated during pilot studies. Discussions need to be held with IERL concerning the availability of resources to have the protocols written and validated during pilot testing.

APPENDIX B

S. Sandhu
EPA/HERL - RTP

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

DATE: December 14, 1978

SUBJECT: Summary of Terrestrial Bioassay Workshop Held at Corvallis, Oregon, November 14-15, 1978

FROM: Shahbeg Sandhu
Biologist, HERL (MD 68)

TO: James Dorsey
Chief, Process Measurement Br., IERL (MD 62)

Dr. Ray Merrill of PMB, IERL-RTP presented the general philosophy for the biotesting of industrial effluents and emissions. He outlined the objectives and approaches for the phased approach for environmental assessment program.

The workshop participants expressed concern for the lack of bioassays in Level I matrix to measure chronic effects. They felt that all the bioassays except Ames test measure the acute toxic effects and that acute effects may not be reliable indicators of chronic effects.

It was emphasized by the workshop participants that although health effects and aquatic bioassays proved to be adequate in pilot studies, there is a definite need for biotesting of industrial wastes using green plants and soil microflora. It was recommended that terrestrial bioassays representing three basic processes in ecosystems i.e. photosynthesis, reproduction and consumption should be included in Level I biotest matrix.

The workshop participants were briefed on the difficulties experienced in the pilot studies in applying terrestrial bioassays, especially those designed to measure the biological effects of gases.

The following bioassays were considered for Level I testing:

Plants

1. Stress ethylene production and foliar injury
2. Tradescantia micronuclei and pollen tube elongation
3. Seed germination and seedling growth
4. Pea tendril coiling
5. Tomato petiole angle measurement
6. Bean hook opening
7. Chromosomal breaks in beans
8. Stomatal movements
9. Pollen germination

EPA FORM 1320-6 (REV. 3-76)

The problem with most of these bioassays except numbers 2 and 3 listed above, is that they have never been used systematically for evaluating the toxicity of complex mixtures. Two among these bioassays were selected for further consideration. These were: 1) seed germination and seedling growth; and 2) stress ethylene production and foliar injury.

The stress ethylene bioassay designed primarily to measure the effects of gases is still under development. The previously approved stress ethylene production protocol included in IERL-RTP Level I Bioassay Manual, required the transportation of large volume of gases into laboratory. The pilot studies performed by IERL-RTP have clearly shown the transportation of large volume of gases to be impractical. In addition, there are serious reservations in the scientific community on the reliability of this bioassay. A new version of this bioassay is under development at the University of Massachusetts. This research effort sponsored by EPA, if sucessful, will eliminate the necessity for transporting large volume of gases from industrial sources into laboratory. However the development and implementation of this bioassay will take two to three years. I do agree that foliar injury test has some merit but its sensitivity to low concentrations of gases has to be verified.

The seed germination and seedling growth bioassay has been used by biologists for the past thirty years to measure the genetic damage caused by radiation and chemicals. It is an inexpensive, rapid, simple and reliable bioassay. However it suffers from the basic limitation of need for transporting large volume of gases into laboratory for seed treatment. For testing the liquid and solid samples, this bioassay appears to be very useful. However, some developmental work on this bioassay is needed, which, I do not believe should be too expensive or should take too long to accomplish. The following areas need to be addressed relevent to this bioassay:

1. Growth medium; i.e. peat moss, sand, etc.
2. Climatic variables; i.e. temperature, humidity, light intensity, etc.
3. Plant species; i.e. lettuce, tomato, grasses, raddish, etc.

One of plant species which may be of great use in biotesting is Arabidopsis. It has very small seeds and short life cycle. I understand that Dr. David Tingey is already looking into the possibility of utilizing this species.

It is unfortunate that Tradescantia micronucleus assay was not given a serious consideration. This bioassay measures the genetic damage induced by environmental pollutants in the germ cells. The preliminary studies performed by Dr. Te S. Ma of the Southern Illinois University, on the diesel exhaust has shown Tradescantia test to be very sensitive for in-situ measuring the effect of gases.

Soil Bioassays

The use of soil microflora for testing the biological effects of industrial wastes is fretted with a lot of problems. The numerous types of soils and variables associated with soil makes it very difficult to implement the biotests based on the use of soil.

After considerable discussion the following soil bioassays were recommended for consideration for Level I biotesting.

1. Seed germination and seedling growth
2. Respiration
3. Nitrogen fixation

The major limitation for these biotests is that although these methods have been known to biologists for a long time, these have never been used for testing the complex environmental mixtures. The appropriate protocol for each of these bioassays has to be developed and validated before these could be used for routine biotesting.

The workshop participants recommended that the methods of extracting and leaching toxicants from soil should be further examined.

Animal Bioassays

Two bioassays based on the use of honeybees and Drosophila were recommended for consideration for Level I testing. Both tests looked very promising.

Drosophila has been used quite extensively for genetic and behavioral studies. Honeybees although somewhat difficult to manage appear to be useful experimental organisms.

Level I to Level II Trigger Criteria

It was suggested that the data from terrestrial bioassays may be expressed in terms of degree of toxicity i.e. not detectable, low, medium, and high. Those samples showing high toxicity should be tested in Level II bioassays. The samples showing less than high toxic response in terrestrial bioassays and showing consistant toxic response in health effects or aquatic bioassays, should be retested using Level I terrestrial bioassays employing additional species of plants and insects. If the effects initially detected are confirmed in other representative species, then Level II tests should be performed on fractionated samples.

Level II, testing will be performed on fractionated samples uisng the same bioassays as in Level I. In addition, the life cycle studies in plants and insects were recommended.

It was recommended that soil microcosms should be included in Level II test matrix.

Dr. David Tingey assumed the responsibility of writing the protocols for the proposed bioassays. It was recommended that the reliability of these bioassays should be evaluated in pilot studies.

In summary the following bioassays were recommended for Level I pilot studies to evaluate the terrestrial effects.

Plants

1. Stress ethylene and foliar injury
2. Seed germination and seedling growth

Soil

1. Nitrogen Fixation
2. Respiration

Animal

1. Drosphila melanogaster
2. Honeybees

For Level II the use of plant life cycles and soil microcosms were recommended. Among all the bioassays included in Level I, terrestrial bioassays appear to be least developed. After pilot studies, no more than three terrestrial tests should be selected for Level I.

APPENDIX C

J. Bromenshenk
University of Montana

Reference: Acurex Project 7347
Contract No. 68-02-2611
Subcontract RB82542A

SUMMARY AND DISCUSSION OF THE TERRESTRIAL BIOASSAY WORKSHOP

DEFINITION OF TERRESTRIAL ECOLOGY BIOASSAY PROTOCOL FOR ENVIRONMENTAL ASSESSMENT PROGRAMS

by

Jerry J. Bromenshenk, Ph.D.
Ecologist/Entomologist/Consultant

733 West Sussex, No. 3
Missoula, Montana 59801

November 25, 1978

INTRODUCTION

The Terrestrial Bioassay Workshop held November 14 and 15, 1978, in Corvallis, Oregon, reviewed the status of the IERL-RTP Environmental Assessment (EA) Methodologies with specific reference to Terrestrial Biotests. The stated objectives were to specify a battery of terrestrial ecology bioassay tests for the Level 1 protocol, to formulate Level 1 to Level 2 decision criteria, and to propose Level 2 terrestrial bioassay protocols. The participants were asked to address the critical question of whether the current protocols proposed for the IERL-RTP terrestrial ecology tests were appropriate and if there were other tests available for use which would be better than those proposed or which also should be included.

The following viewpoints were expressed by workshop participants and are relevant to a summary and discussion of the outcome of the meeting:

(1) The workshop organizers hoped to "cast in concrete" a Level 1 terrestrial ecology bioassay protocol (TEBP) which could then be presented for program review.

(2) While acknowledging the need for specifying a Level 1 TEBP, the attendees stressed that any bioassays proposed during the workshop must be defined as those thought to be the best tests currently available but not necessarily the best tests which could be performed--a major consideration for the tests proposed was that results be reproducible by different laboratories.

(3) The Level 1 biotests, which were recommended, demonstrate only acute effects. But there is no evidence that acute effects are indicative of chronic effects in terms of biological responses. Chronic effects

may be considerably different from acute effects. Also, the biotests proposed for use do not consider latency. Thus, it must be recognized and stated in the IERL-RTP program that these responses cannot be addressed because of a lack of suitable, well-developed, repeatable bioassays which also can meet the stated Level 1 cost and time constraints. The conclusion should be that at present these tests cannot be performed, not that they don't need to be done.

(4) Ideally the decision criteria for specifying the needs for Level 2 terrestrial ecology tests based on Level 1 results would involve unequivocal triggers to determine the necessity of Level 2 testing. However, the consensus of the workshop participants seemed to be that limitations of currently available bioassays and a lack of knowledge concerning the complex ecological effects of individual pollutants, not to mention multimedia, multipollutant streams, requires that a step by step, case by case, decision process by qualified experts be utilized.

(5) The workshop committee felt that they could not set forth a set of perfect Level 1 bioassay tests, nor could they establish, in many cases, easy to follow, clearly-delineated decision criteria for proceeding from Level 1 to Level 2. Despite these problems, the committee supported the inclusion of terrestrial ecology bioassays in the IERL-RTP program. Bioassays give the actual reactions of individual organisms or populations to pollutants, they respond to synergistic, additive, and antagonistic properties of mixtures of pollutants, and they integrate responses through time. Chemical and physical characterization of pollutant streams can attempt only to predict a very limited number of biological responses to relatively few substances for which there is an existing data base concerning effects such as toxicity or mutagenicity. Also, this usually only can be done for individual chemicals rather than mixtures.

WORKSHOP SUMMARY AND CONCLUSIONS

The participants of the Terrestrial Bioassay Workshop recommended a set of specific tests to be used in a terrestrial ecology bioassay protocol for the Level 1 Environmental Assessment Program. Also, they indicated appropriate categories of tests for a Level 2 protocol. The bioassays for Level 1 were chosen keeping in mind the need to evaluate pollutant streams which involves a chemical test matrix of liquid, solid, and gaseous samples. The recomendations for the protocols for Level 1 and Level 2 bioassays were that:

(1) Plant bioassays for Level 1 focus on phytotoxicity and be comprised of: (a) the stress ethylene plant response tests coupled with determinations of foliar injury, and (b) seed germination tests linked to seedling growth observations. Level 2 testing would incorporate fractionation of chemical samples, tests of the responses of additional plant species, and tests which would include full life cycles of plants.

(2) Soil bioassays for Level 1 concentrate on endogenous soil respiration but also include tests of ethylene reduction (an indication of

nitrogen fixation). Possible inclusion of a test of effects on hydrogen-oxidizing microorganisms using the tritium-labeled hydrogen (tritiated water) procedure developed by EPA-Las Vegas was suggested. Level 2 testing would utilize soil microcosms, specific substrate respiration, and presumably fractionation of chemical samples for further testing.

(3) Animal bioassays for Level 1 examine zootoxicity and encompass in vivo toxicity tests using invertebrates in addition to the mammalian bioassays to be performed under the health effects testing program (IERL-RTP Procedures Manual: Level 1, EPA-600/4-77-043, April, 1977). Honeybees (*Apis mellifera*) and/or fruit flies (*Drosophila melanogaster*) were proposed as the test organisms. Level 2 testing would take into account fractionated chemical samples, additional invertebrate species, and other biological responses such as evidenced by behavior, physiology, reproduction, and full life cycles.

The decision criteria for specifying Level 2 terrestrial ecology test needs based on Level 1 results was one of the most difficult issues addressed at the workshop. However, there were several points of general agreement:

(1) It should not be necessary to perform Level 1 bioassays nor to make decisions concerning Level 2 for chemical streams containing substances known to be extremely hazardous or containing unacceptable levels of these substances. For example, a chemical stream with a pH of 2 would be expected to kill just about anything in it, while streams containing quantities of arsenic, cyanide, or mercury would be subject to existing regulations and standards concerning acceptable levels of release of the contaminants and the control of their release into the environment. It is important that the final procedures manual contain a statement about not performing needless bioassays on chemical streams of this sort and provide guidelines towards making this determination.

(2) Ideally, Level 1 results should provide easy to interpret warning flags, yes-no choices, or triggers which would decide both the need for Level 2 testing as well as specify the types of tests to be performed. In some cases, this will probably occur. For example, if the results of all the Level 1 chemical and biological tests indicate no hazard, the decision is fairly straightforward; there is no need to proceed to Level 2. On the other hand, if all the chemical and biological tests indicated that the degree of hazard was high, then one would definitely proceed to Level 2. However, if only some of the chemical and biological tests indicated a possible hazard, if some indicated a high degree of hazard and others low or none, or if most suggested that the degree of hazard was moderate, then the decision criteria would become much more complex and would not be simple or clearly defined.

(3) Obviously, there is a need to prioritize or rank chemical streams in terms of harmful biological (health, ecological) effects based on the Level 1 results. Ranking within a single biotest such as

assigning toxicity values (no detectable toxicity, low toxicity, moderate toxicity, and high toxicity) based on Lethal Dose 50% of specified concentrations to rats (as in Figure 4, page 11, of the Draft document entitled Bioassay Procedures for Screening Complex Effluent Samples) can be easily standardized. But prioritizing and ranking the results of a battery of bioassays is much more difficult and is dependent on a clear definition of goals and an understanding of what the tests results mean.

(4) Limitations of knowledge concerning problems such as substances for which there is little or no data on toxicity or mutagenicity to organisms, synergisms and antagonisms of chemicals which may alter harmful effects, the adequacy and the representativeness of the specified Level 1 tests in terms of the biological effects not only to a few specific systems but also in a more holistic sense, and the weighting of Level 1 bioassay results will necessitate, in most cases, an ad hoc decision process which must be conducted by qualified experts.

During the final session of the workshop, participants expressed concern for quality controls at all stages or levels of the test program from the initial sample collection (including the planning of its collection) through the final data interpretation and reporting. The proposed controls for quality assurance included the use of standard control samples, reference controls, single blind controls, and splits of the samples and tests between laboratories performing the assays. Reference compounds for the toxicity tests should include materials representative of inorganic, organic, and organo-metallic pollutants. Workshop participants from the Corvallis Environmental Research Laboratory had participated in the development of the stress ethylene plant response test and indicated that they had a list of phytotoxic chemicals that could be used as a data base from which to pick toxicity references. For the other Level 1 bioassays, 2-4-nitrophenol was suggested for the soil respiration inhibition test, while chemicals from the pesticide testing programs were suggested for use in the invertebrate tests and included monosodium methane arsenate, an organophosphate insecticide (methyl parathion, preferably Penncap M (R)), and a carbamate insecticide (Sevin (R)).

The need to completely describe the procedures for each of the proposed Level 1 bioassays was recognized, but it was felt that this could not be accomplished at the workshop. There was some concern that the bioassays that had appeared in the previous IERL-RTP procedures manual were not precise enough concerning many of the procedural details.

GENERAL DISCUSSION

Some points concerning the discussions at the Terrestrial Bioassay Workshop deserve further mention.

Levels 1, 2, and 3 were defined and for the most part follow the explanation given in the background information documents provided at the meeting. However, according to at least one of the background information documents, chronic sublethal effects would be relegated to Level 3. I do not believe that the workshop participants were aware of this. There was discussion as to

whether chronic sublethal tests belonged in Level 2. Some felt that inclusion of these tests at Level 1 was desirable, although probably not feasible.

It should be mentioned that in choosing Level 1 bioassays, the workshop group took into account many factors such as chemical matrix (gas, solid, liquid), biological processes (photosynthesis, decomposition, consumption, nitrogen fixation, etc.) and responses (growth, development, reproduction), and procedures (cost, sample type, relevance, availability, comparability).

The stress ethylene plant response test was thought by some members of the group to be overly general, while others were concerned about the ability to quantify foliar injury, since it may be expressed in many different ways (lesions, chlorosis, mottle, necrosis). The idea of coupling the two should offset some of the uncertainties of each taken alone. There was some mention that not all investigators who have performed the stress ethylene test were satisfied with it, but no one at the meeting explained what specific problems had been encountered. Apparently, space was a problem, and a miniaturized version of the test using plants grown on agar in test tubes is being developed. I should think that such a highly artificial system would have to be very carefully evaluated against full size controls in order to determine the effects of miniaturization. Also, there was no mention made at the meeting that foliar injury linked with histopathological examinations of the damaged area can provide considerable information concerning the nature of the damage and may provide a useful "fingerprint" for specific chemicals or chemical classes.

Seed germination and seedling growth were chosen because these provide information concerning two important biological processes--reproduction and growth. There exists an extensive body of knowledge concerning this type of test, and it was surprising that germination and growth tests had not been included in the original bioassay protocol.

Pollen tube elongation and pollen viability were favored by some members of the committee but were excluded because of problems concerning pollen viability during storage and the interpretation of the results.

Tradescantia was suggested as a potentially valuable test organism for mutagenicity in higher organisms (eukaryotes as compared with the prokaryotes used in the Ames test). The *Tradescantia* assay involves a color change of cells of the stamens or filaments of the flowers. Although it was not brought out at the meeting, the main problem with this method may be that large numbers of cells must be counted to give significant results, especially if the chemical dosages are very low (a background incidence of the color change must be taken into account).

Several other plant bioassays were proposed and discussed, but none seemed to offer as much potential as those chosen, or the tests were not as well developed.

Since the soil microcosm test was the only Level 1 test above a species level, it received considerable attention. However, the sensitivity of the system was considered to be low, and the test appeared to be data rich but difficult to interpret. The test probably would be difficult to standardize because of chemicals already present in the soil cores. There is a question of which soil types to use and a problem of how to obtain a standard core.

On the other hand, procedures such as soil respiration have had many years of usage, and these procedures have been standardized and are considered to be reliable. Several alternative or additional tests were advocated, including a Mason jar soil CO_2 test, nitrogen fixation, nitrification, biodegradation profiles (starch, cellulose, protein, pectin), and hydrogen-oxidation (tritiated water). Many of these seemed to have merit, and it was suggested that they be examined for possible inclusion at a later date. Soil microcosm testing was moved to Level 2, since it tends to be data rich. It was felt that this data richness might tend to send everything to Level 2, whenever the test was performed.

During the discussion of the animal bioassays, concern was expressed that many major groups of animals, such as birds and invertebrates, were not represented. No one at the meeting was prepared or qualified to indicate an appropriate bird test. However, insects were deemed to offer considerable potential as bioassays. There appeared to be mutual agreement that insects should be included, and the debate turned to which species to use. Honeybees and fruit flies were given priority. I shall discuss this topic more completely in the specific discussion section of this paper.

Quality assurance was recognized as an essential aspect of the bioassay program. Care must be taken to insure that quality assurance is built into the protocols. Problems such as the effects of sample integrity and age, effects of collection procedures, the form in which chemicals should be tested, the need for the testing of chemical fractions in Level 2, whether Level 2 requires new or additional chemical samples, and the need to concentrate samples for testing were discussed on a case by case basis. However, quality assurance and these other problems require that qualified experts be involved not only in the development of the procedures manual but also during the testing. There must be cooperation and involvement by biologists and chemists during all stages of the program to insure its validity and success.

SPECIFIC DISCUSSION

In this section, I shall confine my remarks to my personal expertise, i.e., environmental entomology.

There are several programs that already require or will soon require testing of pesticides and other toxic substances on honeybees because there have been substantial losses of bees in recent years attributable to the use or misuse of insecticides. This in turn affects the products and services provided by bees--honey, wax, and pollination. In addition, honeybees have been tested and rated according to their sensitivity to hundreds of chemicals. Toxicity testing procedures for bees are well-defined. Also, honeybees appear to be more susceptible to harm from many toxic chemicals than other animals including man. There is a chance, although slight, that chemical toxins may pass to human food chains through honey.

Since a queen honeybee may live for several years and is the only reproductively mature member of a colony, resistance to hazardous substances is unlikely to evolve, although resistance to pesticides, for example, has evolved rapidly in many other insect species. Since all worker bees in a colony are haploid offspring of an individual queen, genetic variability can be limited, and controlled breeding can be used to convert bee colonies to the "same" genetic composition.

Finally, honeybees not only could be used in a variety of ways in a Level 1 and a Level 2 protocol, but they could be used in a Level 3 protocol. In fact, a few monitoring programs in the United States and Europe presently are using honeybees in a manner representative of the projected Level 3 protocol.

Conversely, *Drosophila melanogaster* is a good test organism. Extensive genetic research has been conducted with it. This insect also has been used in bioassays of pollutants. It would be difficult to say whether there is a more extensive body of information concerning *Drosophila* than *Apis mellifera* but suffice to say that both have been studied over a very long period of years.

Drosophila, at first glance, would appear to be somewhat easier to rear and maintain than honeybees. But once an apiary has been established, honeybee colonies are more or less self-sustaining and require very little care. Honeybees tend to be more vigorous if the colonies are kept outdoors and the bees are free to fly. They can be brought into the laboratory for testing, and also they can be tested in the field (field testing using *Drosophila* would be less convenient). In order to be able to conduct bioassays all year round, a laboratory doing honeybee bioassays probably would have to be located in the south or in a coastal area of the United States--areas with warm winters.

The preceeding summarizes comments regarding insects made at the Corvallis workshop. The following comments reflect my own opinions and may not represent those of the other members of the workshop committee.

Chemicals other than pesticides have been shown to have significant effects on many terrestrial insect systems. These include gaseous, liquid, and solid chemical forms and include smog, ozone, hydrocarbons, nitrous oxides, fluorocarbons, sulfur oxides, dusts, major and trace elements, radionuclides, and acid mists. Routes of entry into the body of an insect may be by inhalation, injestion, or penetration through the cuticle. Biomagnification may occur as toxic materials are passed along insect food chains.

Biologically active pollutants have been shown to affect the biochemistry, physiology, and behavior of insects. Toxins may produce both lethal and sublethal effects such as shortened life spans, reduced hatchability, lowered fecundity, genetic alterations (mutations, teratorlogies), toxicant avoidance, disorientation, memory loss, and temporary and/or permanent behavioral modifications. Thus, effects may occur at any level of organization from the biochemical to the ecosystem.

I have attached to this report two papers (References 1 and 2) which reference my comments and provide a more detailed discussion of pollutant-insect interactions.

It should be noted that honeybees and fruit flies are not the only insects that have been studied as regards hazardous chemicals. There exists a large quantity of information concerning insect pests of forests and agriculture, butterflies (industrial melanism), ground-dwelling beetles (predators and saprophages), mosquitoes, and soil arthropods.

The workshop left open the decision of whether to use honeybees or fruit flies or both. Each species has its own unique advantages and disadvantages

for use in a bioassay program. It may well be that each may be used but for different tests, either in Level 1 or especially in Level 2.

During post-workshop inquiries, I found that bioassay protocol using honeybees is being put together for EPA's Pesticide Hazard Evaluation Division. A workshop was held about three weeks before the Terrestrial Bioassay Workshop. The outcome of that workshop was the framework for a screening protocol concerning the effects of pesticides on honeybees. This protocol should be available for comment by February, 1979. Information concerning this program can be obtained from Allen Vaughn, OPP, EEB, HED, Room E 107, TS 769, 401 M Street SW, Washington, D.C. 20460, telephone (202) 426-0224.

Briefly, the proposed pesticide protocol involves acute toxicity, dose-response, linear regression curve determinations using caged bees in the laboratory or possibly colonies in the field. The procedures have been developed and tested by E.L. Atkins and associates at the University of California, Riverside, and by R.J. Barker and associates at the USDA-ARS Bee Research Laboratory, Tucson, Arizona.

A second set of tests to be used will examine sublethal or chronic effects which may be disruptive to the social organization of bee colonies and which could be far more important in terms of harm than mortality per se. The projected tests would consist of 60-day tests, probably carried out in a screened greenhouse. All experiments would start with clean equipment. The choice of the number of bees and the type of hive used would be left, within certain guidelines, to the investigator. At 30 days and at 60 days, the amount of brood would be determined by measuring the comb area covered by brood. At the end of 60 days, determinations such as the weight of bees (indicates number) and the weight of the product (honey) could be made. During the run period, observations could be made concerning rates of mortality (as measured by dead bee traps), abnormal adult behavior, morphogenic changes, etc. Controls and standards would be utilized where appropriate.

Although this protocol is being developed with reference to pesticides, it should be easy to adapt to other hazardous chemicals. Liquids and solids could be administered in controlled dosages in water or food (honey, pollen) or could be misted or dusted onto the bees. Gases would require some type of fumigation apparatus similar to that needed for the plant bioassays.

Drosophila melanogaster is a very useful organism for genetic and life cycle studies, although possible evolution of resistance would have to be continuously monitored. *Drosophila*'s life span is relatively short. Rearing procedures are standardized. A fumigation apparatus using half pint milk bottles (the standard rearing containers) has been developed by M.E. Ginevan, Argonne National Laboratory, Argonne, Illinois 60439, and D. Lane, Department of Civil Engineering, University of Kansas, Lawrence, Kansas 66045. These investigators have conducted effects tests of sulfur dioxide over the life cycle of the flies and on specific life stages (egg, larvae, adult). They carried out experiments over periods from 4 to 20 days using continuous, "low" level (0.4-0.7 ppm) sulfur dioxide fumigation. The types of responses that they quantified included increases in development time, decreases in survival, genetically-controlled differential treatment response, and reduced activity levels (torpid, fed sporadically).

The workshop committee recommended that the Level 1 insect bioassays concentrate on in vivo toxicity. However, since both *Drosophila melanogaster* and *Apis mellifera* have short life cycles, it is feasible to incorporate determinations of effects such as shortening of life spans simply by continuing the toxicity evaluations for a period of three weeks. *Drosophila* has a generation time of about ten days, while honeybee queens develop from egg to adult in approximately 15 days, workers in 21 days, and drones in 24 days. The queen mates 5 days after the imaginal molt, and she begins to lay eggs 2 days later. Shortening of lifespans of honeybees affects honey production since it is the oldest bees that do most of the foraging.

Either in Level 1 or Level 2 if a sufficient number of graded dosages were administered, one could perform linear regressions or probit analyses of dose-response. This information could be applied towards making predictions (restricted to insects) about the possible consequences (toxic effects) of releasing specified amounts of the chemicals of concern into the environment.

Suggestions made at the workshop for Level 2 insect bioassays included fractionation of chemicals for toxicity and other types of evaluations, the use of additional insect species, and the assessment of effects on full life cycles, reproduction, and behavior.

Drawing from experience in pesticide testing, additional insect species might include mosquitoes, aphids, crickets, or grasshoppers. Mosquitoes are very sensitive to many toxins, often more so than even honeybees or fruit flies. Crickets are convenient test animals and some species have a pest status. Grasshoppers and aphids are two major pests that insecticides are designed to control. Any number of other major pest insects such as those of forests, croplands, orchards, or gardens could be used. The insecticide industry conducts tremendous numbers of tests concerning the efficiency and mode of action of their products on target organisms.

I should think that a mutagenicity assay using *Drosophila* would be appropriate for Level 2. Like the proposed *Tradescantia* mutagenic test, this would provide information specifically concerning higher (eukaryotic) organisms, unlike the Ames tests that focuses on bacteria (prokaryotes).

Another type of test which might be considered for Level 2 is a morphogenic test. For example, E.L. Atkins and associates at the University of California, Riverside, have been investigating morphogenic responses of honeybees to pesticides. In this test, the chemical(s) is inserted with a micro-pipette into brood at different concentrations and at different stages of the insects' development. As the adults emerge, the number dead, deformed, underweight, etc. are recorded. The results are then correlated with dosages and stage at which the chemical was introduced. Thus, the test provides an assessment of interactions with growth and development.

Insect behavior could be a very sensitive and very rapid bioassay and would provide information not given by any test other than the aquatic effects assay using *Daphnia*. Abnormal web spinning by spiders (Arachnids) has been used by pharmaceutical companies in evaluations of the effects of certain drugs. The rate of singing or chirping by crickets is altered by chemicals and other environmental stresses. Honeybees demonstrate disorientation, losses of memory, or impaired locomotor activity in response to sublethal

dosages of insecticides and chemicals such as carbon dioxide and nitrous oxides. Pence and Lomax (Biodynamics of the Excised Honey Bee Abdomen. Insect World Digest 1(1):16-24, 1973) reported that the movements of excised abdomens of bees can be used as signatures for specific pollutants. They have performed the procedure for odors, gases, polluted water, and pesticides in soils.

Effects on reproduction such as altered fecundity and offspring viability seem to me to be part of life cycle observations and not a separate category.

The Level 2 tests that I have indicated are meant to be illustrative and not an attempt at a comprehensive treatment of the subject. Any number of potentially useful and informative bioassays of chemical streams could be performed using insects or other invertebrates. I believe that insects offer many advantages as test organisms in environmental assessment protocols because of their abundance in species and numbers, small size, short life spans, importance to ecosystems, sensitivity to environmental perturbations, and history of use in evaluations of chemicals in pollution monitoring and in ecological studies.

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APPENDIX D

K. Duke
Battelle-Columbus Laboratories

SUMMARY OF
EPA TERRESTRIAL BIOASSAY WORKSHOP, NOVEMBER 14, 15, 1978

Summary

The EPA Terrestrial Bioassay Workshop was held in Corvallis, Oregon November 14 and 15, 1978, under the direction of Acurex Corporation. The workshop focused on the phased approach to biological testing of complex effluents from energy and industrial processes and had three objectives:

1. Develop recommendations for Level 1 terrestrial bioassays
2. Develop Level 1 to Level 2 decision criteria
3. Suggest potential Level 2 testing procedures.

Participants included professional researchers familiar with terrestrial bioassay procedures and/or EPA's phased approach to testing.

The summary presented here is taken from the author's notes and represents his understanding of the conclusions reached at the workshop. The author's comments on these conclusions is presented in a separate section of this report.

Level 1 Test Procedures

Level 1 testing is the initial screening level of the phased approach. Two terrestrial tests, plant stress ethylene and soil microcoms, had been originally proposed for the bioassay protocol. However, EPA pilot studies had revealed some problems with these tests. One objective of this workshop was to develop recommendations for Level 1 terrestrial tests which would meet Level 1 criteria and the acceptance of the EPA Bioassay Subcommittee and other technical experts. In developing the recommendations at least six criteria were considered for each candidate test: (1) cost, (2) sample requirements (form and quantity), (3) relevance to terrestrial ecology and biology, (4) availability (existence of accepted test procedures and a data base developed from previous use of test), (5) comparability (among different labs performing the test), and (6) response (easily measured and sensitive).

Four candidate tests were suggested for the plant (photosynthetic) aspect of the terrestrial environment. They were foliar injury/stress ethylene, Tradescantia mutogenicity/toxicity, seed germination rate and seedling growth and dvelopment (several specific tests). Three of the four tests (Tradescantia was dropped) passed the screening and were recommended for Level 1 tests.

A second aspect of the terrestrial environment is the process of decomposition in the soil. This process is important because it is key to nutrient recycling in ecosystems. Numerous suggestions for suitable tests were made including several bacterial tests, nigrogen fixation, sludge testing, and the soil microcom tests. Two tests, ethylene reduction and total endogenous soil respiration, were finally recommended for Level 1.

Animal (consumer) tests suggested for consideration were primarily acute toxicity tests using mosquito larvae, Drosophila, honey bee, and others. The honey bee/Drosophila actue toxicity tests were finally recommended.

Level 1 to 2 Decision Criteria

While some time was spent discussing this topic, no definitive recommendation was made. The results from Level 1 testing--biological and chemical--are needed to priortize the waste stream tested. Those high in priority are subjected to Level 2 first. Medium priority streams are tested later if time, money, sample quantity, etc. permit. Lowest priority streams, while not eliminated out of hand from further testing, are not likely to proceed to Level 2. It was expressed at workshop that streams obviously toxic in both chemistry and biology and those which showed no toxicity could be easily prioritized. It was the ones with indeterminant results that would provide the most difficulty in accurately assigning priorities. However, no recommendation was developed for these "grey" area streams.

Level 2 Test Procedures

Recommendations for potential Level 2 plant tests were of two types. The first recommendation was to retain the same tests as used for Level 1 but to use different species. New tests which could be used at Level 2 were the Tradescantia mutogenicity/toxicity test and numerous

growth and developmental tests such as pea seedling growth, bean hook opening, and others. A terminal bud genetics test was also suggested. All tests suggested will require further developmental work to be suitable for Level 2 use.

Level 2 tests suggested for the decomposition process included the soil microcosm and specific substrate respiration tests. Again, developmental work will be necessary before they can be actually implemented.

The suggested animal tests for Level 2 focused on life cycle and behavioral tests using various different species. These types of tests are directed at long term effects (life cycle) and subtle subacute (behavioral) responses which are often very sensitive. Some developmental work would be required for these tests before incorporation into Level 2.

Comments

Level 1 Test Procedures

In the author's opinion those tests recommended for Level 1 comprise an adequate protocol capable of meeting the objectives of the phased approach. This protocol is definitely superior to previous Level 1 terrestrial scheme. Some of the problems remaining with the newly recommended protocol include the use of tedlar or teflon bags (needed because of the large volume required) to collect the gas sample for the stress ethylene test. Some of the constituents of the gas are known to either adhere to or pass through the walls of the bag altering the nature of the sample that will be tested. The constituents so lost may or may not be toxic ones of interest. In spite of these sample problems, stress ethylene test is still of use in Level 1 because of its high sensitivity and relatively linear dose response curve. The sample problem is partially offset by the data obtained through on-site gas chromatography for Level 1 chemistry and the decomposition and animal tests on gases which, because of the small quantity needed, can be collected in glass containers (where adherence and permeation are not a problem).

Another aspect involving the Level 1 animal tests deserve some comment. No final decision was made as to which of the two insect species, honey bee or Drosophila, should be used in the acute test. At present, I would

recommend Drosophila in spite of the compelling argument for the honey bee based on its great economic value. The Drosophila are smaller requiring smaller sample quantities (expecially critical for particulate and gaseous samples). They are easier to handle because they don't sting. They can be maintained year round in laboratories under constant environmental conditions. Honey bees are more often reared outside and become less active in the winter which can alter their response in acute bioassays.

The endogenous soil respiration test is comparatively less sensitive and more variable than some of the other bioassays recommended. This variability problem must be recognized when using the test results. This test still remains an important and useful one in that it involves a critical component of terrestrial ecosystems for which data are needed at Level 1 to aid in effluent prioritization.

Performing Level 1 bioassays on-site especially for those such as the stress ethylene test where sample quantity and quality are a problem has been suggested. I believe the additional cost, quality control, and logistics problems of on-site testing far outweigh any advantages obtained by such testing. This is particularly true at Level 1 where the tests need to be cost effective, quick screens for toxicity rather than expensive, more definitive procedures. Off-site laboratory tests are fully adequate for Level 1 testing and probably Level 2. Only Level 3 may need on-site tests.

One final area of comment for Level 1 is the need for further test development. While the tests suggested are adequate for Level 1, there are many others which show promise of being even better (cheaper, more reliable, more replicable, smaller sample requirements, etc.) but need some additional developmental work to make them acceptable. This developmental work involves both refinement of test procedures and validation of the test in actual routine usage. Such developmental work will be slow or lacking altoghther unless the interest in this work is organized into some type of test development program. Since the development of these tests has value to both IERL-RTP, other EPA ORD groups, and the EPA program offices responsible for implementing various environmental laws (e.g., TSCA, RCRA), some type of coordinated jointly funded effort should be possible and certainly desirable.

Level 1 to 2 Decision Criteria

The author believes this to be one of the most important difficulties to be solved in the phased approach. There is little experience in synthesizing both Level 1 chemical and biological data to obtain a realistic ranking of waste streams. Streams showing high toxicity or no detectable toxicity in Level 1 biological tests and revealing significant quantities of many known toxic substances or none in Level 1 chemistry are easily prioritized. It is the test results in between these two extremes that present interpretation problems, i.e., samples containing no significant quantities (detectable with Level 1 chemical procedures) of known toxicants which give toxic responses in several bioassays or samples giving toxic responses in some biological tests and nontoxic responses in others. This problem is compounded by the fact that there is a potential for false negative test results and that the tests have varying sensitivities. Varying sensitivity means that if higher priority is placed, for example, on a relatively insensitive health test (because it is indicative of effects on a man) rather than a very sensitive terrestrial test, it is possible that a waste stream containing a toxic chemical may be given a lower priority than it deserves. Until our knowledge about the correlation between laboratory test results and effects on man is improved, the weighting of one piece of Level 1 information over another should be done with care. Some system to use all Level 1 data is needed. Suggestions for using such data include:

1. A "high" (as per the Litton evaluation scheme) in any test should automatically raise a stream's priority
2. A "medium" both in one health test and one ecological test should raise a stream's priority
3. A "low" in one health test, one aquative ecology test, and one terrestrial ecology test should raise a stream's priority.

As the test results for Level 1 bioassays accumulate for different kinds of samples, it may become apparent that some of the tests are particularly sensitive and reliable and others are insensitive or variable. At that time, it may be appropriate to either revamp the test protocol or weight the sensitive, reliable test results higher.

In interpretation of test results, the use of the "degree of hazard" method and the MATE or MEG values should be approached with caution. Level 1 chemistry does not provide data fully compatible with MEG and MATE values leading to misleading rankings of waste streams. This was demonstrated by the Level 1 pilot study results.

Level 2 Test Procedures

Level 2 recommendations resulting from the workshop carry the necessity for further test development. My comments on this need have already been given in my Level 1 comments. Coordinated developmental efforts are needed if effective Level 2 procedures are to become available.

A final suggestion for Level 2 concerns the philosophy and the procedures to be used. Level 2 has two objectives: (1) to confirm Level 1 results and (2) to isolate the toxic chemical(s) through fractionation and retesting. High priority streams receiving bad marks in several Level 1 chemical and biological tests should not need confirmation. It should be possible to go directly to fractionation. Medium priority streams should need confirmation. This confirmation may be either using Level 1 tests on different test species, using completely new tests, or both. Once toxicity has been confirmed, chemical fractionation and testing should follow. Level 2 biological tests on the fractions could well be the same as used in Level 1 since the objective of testing the fractions is similar to Level 1, i.e., to determine which of the fractions is toxic. Some refinement in Level 1 procedures (e.g., altering the range or number of doses, etc.) may be appropriate to better identify the toxic response. However, it is unlikely that major test changes would be needed for most areas of biological testing.

APPENDIX E

D. McCune
Boyce Thompson Institute for Plant Research

REPORT TO ACUREX CORPORATION ON TERRESTRIAL BIOASSAY WORKSHOP IN CORVALLIS, OREGON ON 14-15 NOVEMBER 1978

1. Summary

The general conclusions reached by the workshop were that there are now suitable candidates for bioassays in Level 1 screening for plant, soil, and animal components of the terrestrial system. It was also concluded that other sets of protocols can be recommended for further investigation and possible development as Level 2 bioassays.

A consideration of the problems involved in biological as well as screening protocols led to the conclusion that decision-criteria for transition from Level 1 to Level 2 screening in terrestrial systems are not independent of but contingent on the results of sampling, chemical analyses, and bioassays for toxicity in aquatic and mammalian systems.

Throughout the discussions, it was evident that many lacunae are present in Level 1 protocols as regards their description, relevance of results to intended use, and quality of data derived from screening procedures.

2. Protocols for Level 1 Bioassays

The following protocols were recommended for bioassays in the terrestrial ecology systems on the basis of three kinds of criteria. Firstly, they represented significant components (receptors) of the terrestrial system and their responses had relevance to possible adverse effects. Secondly, the bioassays generally were appropriate to stream-classifications, i.e. gas, liquid, or solid, with respect to the material to be assayed. Thirdly, the bioassays were among the most practical in terms of availability, cost, efficiency, etc.

2.1. Animal Bioassays

Two test systems were recommended and differ mainly in the organism to be used: honeybee (*Apis mellifera*) and fruit fly (*Drosophila melanogaster*).

2.1.1. Protocols

Specifics of these test protocols were not formulated at the workshop, but were to be formalized later.

2.1.2. Rationale for Selection

The following kinds of considerations entered into the selection of these bioassay systems.

(a) Cost. Well within appropriate range.

(b) Availability. Stock cultures of organisms are easily and economically maintained and numerous sources are available. Moreover, protocols for the exposure of the organisms have been developed, and extensive background data of their genetics, tolerances, and behaviour are available.

(c) Sample suitability. The particular stream-phases to which this test would be applied were not specified. But it appeared that gas-, liquid-, or solid-phase materials, in the amounts to be obtained, could be accomodated because the materials could be administered topically or by ingestion.

(d) Relevance of responses. Definite endpoints of each system could be achieved in terms of morbidity, aberrant behaviour, mortality, or bio-accumulation. Further, these responses would be relevant to environmental effects.

(e) Comparability. Suitable standards are available for quality control and assurance. In the honeybee system, methyl parathione and monosodium methyl arsenate were suggested as standards.

2.2. Soil Bioassays

Two systems were recommended: endogenous respiration and acetylene (C_2H_2) reduction. Both are process-oriented and use the same general test system but differ chiefly in the response measured. Of the two processes, the former is a generalized index of activity whereas the latter is a surrogate measure of nitrogen-fixation.

2.2.1. Protocols

Specific protocols were not recommended at this time but details can be made available. It was proposed that three concentrations with four replicates together with appropriate controls and standards for quality control and assurance are accomodated by the procedure. One difficulty will be in the selection of a standard soil or the kinds of soils to be used.

2.2.2. Rationale for Selection

The following factors entered into the selection of these systems:

(a) Cost. The cost per test would probably be no greater than $500 per stream-sample.

(b) Availability. The procedures are off-the-self and a data base is available that allows judgements as to the sensitivity, specificity, and precision of measurements.

(c) Sample. Sample requirements appeared to be non-critical for liquid or solid streams. Also, sample integrity appeared to present no problem.

(d) Relevance of Response. Sample induced changes in the time-course of endogenous respiration would indicate a general change in the biotic processes of the soil, which could be indicative of an adverse effect on any of many possible organisms or on their interaction. The acetylene reduction would be much more specific as to target and process affected because of its use as a surrogate measure for nitrogen-fixation. Both false positives and false negatives may be high in endogenous respiration and of unknown frequency in the acetylene reduction system.

(e) Comparability. It was noted that the endogenous respiration test is relatively precise with coefficients of variation of about 1.6%. It was also noted that sources of acetylene must be screened before acceptance for use in the test. Suitable standards are available for quality control and assurance: aqueous solutions of silver or cadmium salts, sodium azide, or 2,4-dinitrophenol or gaseous ethylene oxide in the endogenous respiration; sodium azide in the acetylene reduction

2.3. Plant Bioassays

It was agreed that algae cannot serve as surrogates for terrestrial plants and therefore suitable bioassay system that employs higher plants was needed. The stress ethylene test was recommended for use subject to clarification and refinement of the description published in EPA-600/7-77-043 and some caveats concerning its utility and relevance.

The plant bioassay system poses problems, of which some are local and others global with reference to the phased approach.

2.3.1. Sample

It seems to me that the greatest problems in the utility of this system arise in the matter of the sample's integrity, amount, and relevance to control of process streams. From these standpoints the plant bioassay system may be subject to false negatives.

(a) Integrity. The principal problems lie in the possible processes in the sample once it has been obtained; its sorption on or reaction with the walls of the container; gas phase or heterogeneous reactions or condensation in the sample itself owing to the continued concentration of materials and lower temperatures during the interval between sampling and bioassay. Thus it is entirely possible that the bioassay will be conducted with a toxicant of composition different from what is obtained by chemical analyses. It is worth noting that the absence of some processes, such as photochemical transformations, in the atmosphere, may also bias the results of the bioassay.

At present, only two approaches seem to be available to check on the integrity of samples. Firstly, gaseous samples of known

phytotoxicity could be run through the sampling system as standards for quality control and assurance. Secondly, Level 1 chemical analyses could be used to determine the possibility of those processes that would adversely affect the integrity of the sample or possibly to resynthesize the effluent at the point of bioassay. Some discussion was devoted to the possibility of development of a field-bioassay system. However, the scale and need for precise environmental controls in the stress-ethylene test renders it unsuitable for field use.

(b) Amount. The amount of sample available appeared to be almost as limiting a factor as integrity. Aside from bioassay in the field, the only other possibilities were to increase the size of the sample by increasing the volume of the container or by physical concentration of the sample and then revolatilization. The last possibility would gravely affect the integrity of the sample. At present the only answer appeared to be to obtain as large a sample as was practical and economical.

(c) Relevance. Even if sufficient sample of unquestioned integrity were obtained, there would still be the question of reference of the gaseous process-streams to their possible environmental effects for two reasons.

Firstly, stream classification, sampling, and physical and chemical analyses are process- and not receptor-oriented. This results in the problem that one cannot always have a direct mapping of a stream or its components on the possible receptors. For example: the classification of streams as gas, liquid, or solid apparently does not account for the possibility that the atmosphere will deliver components derived from liquid streams, through evaporation of ponds, or from solid streams, by re-entrainment of particles, to the terrestrial system as though they were originally in the gaseous streams.

Secondly, it would appear that whereas the Source Assessment Sampling System (SASS) is suited to the sampling needs and physical and chemical analyses of Level 1, it is more refined than is warranted by the bioassay (on plants) at Level 1. That is, it is possible that coarse and fine particles by themselves or interactively with the gaseous components could be active in the plant bioassay and should be relevant to any environmental assessment.

2.3.2. Protocol

Two principal points of discussion, which were somewhat related to the protocol for this bioassay, were whether this test was sufficiently robust to be useful and whether alternatives were more suitable. With reference to the latter point, it was concluded that other bioassays are available but not of off-the-shelf availability and might be better suited to Level 2 procedures. With reference to the former point, the test appeared to be robust because similar results were obtained by Battelle-Columbus and Corvallis Environmental Research Laboratories. Nevertheless, it seems as though the protocol could be more specific in its descriptions of some details and consider the cost-effectiveness of the experimental designs.

(a) Culture and maintainance of plants. 'Dare' soybean should be used, and in selection for uniformity and size a phenological as well as a chronological age should be specified so that tissues present represent the same physiological range of ages and thereby the same potential range of susceptibilities. It was also suggested that due emphasis be placed on the necessity for uniform and reproducible conditions under which the plants are to be grown. This includes the medium or artificial soil in pots and supply of mineral nutrients and water as well as temperature, humidity, light intensity and quality, and photoperiod. In short, one needs uniform plants that are not subjected to environmental stresses for precise and sensitive bioassays.

(b) Measures of response. It was recommended that the occurrence (with respect to kind, frequency, and severity) of foliar injury be a co-equal measure of response with ethylene evolution. This was recommended not only because necrosis biases the ethylene evolution but also because the occurrence of foliar symptoms is the most documented response of plants to air pollutants and has some relationship (but not an invariant one) to growth and yield. Thus it can be used as a measure of relative toxicity and potential effect.

(c) Dose-Response. There is some doubt in my mind as to how useful the dose-response or range-finding approach will prove in this or the soil bioassay system -- except to test two hypotheses: (1) montonicity of response; (2) greater than (synergistic) or less than (antagonistic) additive affects of the components of the sample. In the protocols for aquatic or other systems this facet of the dose-response situation does not seem to be addressed, and if it isn't, calculations of EC_{50}'s may not be correct and indeed irrelevant. It seems as though some caveat should be given.

2.3.3. Cost and Comparability

It appears that for this bioassay the fixed costs would be relatively great owing to the need for certain facilities in which to grow and test the plants as well as the need for personnel to acquire the expertise required. Once a laboratory has the capability for this bioassay, marginal costs should be relatively slight if plants can be produced on a routine basis and not started and stopped in response to irregular requests for bioassays or shipments of samples. That is, economies of scale can be achieved only within a laboratory unless other facilities have soybeans under routine production for other purposes. Unlike tests for mutagenicity or mammalian toxicity, the plant bioassay system is not widespread. Thus, if samples are to be screened routinely by (for example) two laboratories, the use of a third as an occasional referee may involve extraordinarily high costs.

It would follow that the costs of Level 1 screening and quality control and assurance would become more favorable the more concurrence is achieved within the Offices of EPA as to the need for and nature of a suitable plant bioassay.

As was stated above, a standard material such as chlorine (Cl_2) to be used in quality assurance, could be used alone but also as

an added component (spike) to a sample as it is being taken as a test of sample integrity and the ability of the sample-bioassay system to respond to it.

Given the nature of the sample vis a vis the problem of environmental assessment, one would expect false regatives to be much more frequent than false positives.

3. Possible Protocols for Level 2 Bioassays

A considerable body of discussion developed in response to the problem of what criteria should be used in the selection of tests and what tests best fit these criteria. It also appeared that the tests to be used at Level 2 cannot be arrived at independently of what criteria will be used to decide to go to Level 2-testing. Certain tests were proposed for Level 2 in that their possible development and suitability be explored. Other tests were mentioned as possibilities, as yet more remote, for the terrestrial system.

3.1. Animal Systems

Although the protocols to be used were not specified, it was felt that the fruit fly and honeybee systems could be used for this phase of biological screening. A system with mosquito larvae was also suggested. Both systems could be reduced in scale, if necessary, to what would be necessary for the reduced sizes of samples that would be coming from the Level 2 chemical fractionation of effluent streams. No measures of response were decided upon apart from the general desire to have exposures and measures encompass the life-cycle and monitor behavioural characteristics.

3.2. Soil Systems

Three test systems were proposed as possibilities for Level 2 bioassays. The soil-core microcosm and substrate respiration were suggested as major tests and nitrification was suggested as a possible back-up bioassay.

One theme, which was often implied but never stated explicitly throughout the discussions, was apparent in points raised for discussion of soil-tests: concurrence of bioassays in Levels 1, 2, or 3 with those mandated or to be mandated in connection with the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) or the Toxic Substance Control Act (TOSCA). This point was not resolved but a general impression was that some degree of concurrence would be worthwhile from the standpoints of both efficiency of bioassay systems and planning for possible regulatory actions.

The major criteria that led to the choice of these bioassay systems were: (1) the tests would give a composite or integral measure of effects on the three major functional components of soil, i.e. producers, consumers and decomposers; (2) the tests would give more specific profiles as to the effects of toxicants on soil-processes;

(3) the techniques, protocols, and baseline data are available and minimum amount of work need be done to bring the techniques "off the shelf".

3.2.1. Soil microcosm

Three major considerations that favored the use of this bioassay at Level 2 instead of Level 1 appeared to be cost, specificity, and time required (> 6 weeks). The cost was uncertain but estimated to be in the range of $1300 to $2500 per test. The specificity of the test would suffer if the effluent stream contained the same materials, such as calcium (Ca), sulfate (SO_4), phosphate (PO_4), dissolved organic carbon (DOC), or bicarbonate, as those whose mobility from the soil was to be measured, and the occurrence of false positives and false negatives was unknown. Thus, the test seemed better suited for Level 2 where chemical fractionation is present and the time and cost of the tests are more comensurate with the specifity and richness of the data obtained.

It should be noted that more needs to be specified as to what soil(s) and the composition(s) of simulated rain are to be used in this test.

3.2.2. Substrate respiration

In this bioassay system more specific information would be obtained on biotic processes and thereby it appeared to complement the soil microcosm wherein physical and chemical characteristics, mobility of toxicant, and general effects on abiotic and biotic processes were measured.

Within this bioassay, the effects of a toxicant on respiration would be measured (on a time-course?) with cellulose, starch, pectic substances, or protein as substrate. It was estimated that the test would cost about $500 per substrate and require about 15 days for completion. The occurrences of false positives and false negatives were unknown. The kind(s) of soil to be used was not recommended insofar as its physical, chemical, and biological characteristics were concerned.

3.2.3. Other bioassays

Inasmuch as certain key functions of soil were to be used as measures of response (endogenous respiration and a surrogate of N-fixation of Level 1, and substrate respiration at Level 2), it was felt that nitrification should also be considered, at least at Level 2.

Another consideration, with reference to Level 2 where more would be known of the chemical composition of toxicants, was that degradation or fate of effluents might be considered as part of a bioassay system.

3.3. Plant systems

Several criteria were explored with respect to the kinds of bioassays that could be suitable for Level 2 screening. Perhaps the three major considerations were that: (1) the test be an efficient yet rich source of information as to probable environmental effects; (2) the test exploit what was found in Level 1 sampling and physical and chemical screening; (3) the test be commensurate with other Level 2 procedures in yielding data that could be used in planning, regulation, and control. Three kinds of bioassays were proposed.

3.3.1. Primary

One recommendation appeared to be that the same bioassays be performed at Level 2 as at Level 1 but that they be expanded in scope in two different ways: chemically and botanically.

Firstly, the Level 2 test should be performed on fractions of the sample, which chemical analyses and extrinsic information may show to have the greater potential toxicities. Secondly, the tests should be performed with additional species of plants that were subjected to the toxicant for the full life-cycle (possibly a system with Arabidopsis could be developed for this purpose). The desire to broaden the range of test species was based on the knowledge that a considerable variability in tolerance to any compound exists between and even within species. The extension of tests over a larger time period was based on the knowledge that chronic as well as acute effects are possible and that in any plant, certain stages of growth or reproduction are more sensitive to pollutant-induced effects. There was also the consideration that a definite and meaningful end-point could be achieved if the yield of biomass or seed were used as a measure of response.

It should be noted here that whereas temporal patterns of occurrence are placed in Level 3 sampling and analysis, these data are of great relevance to whatever plant bioassays are to be performed and certainly should be in hand to plan Level 3 bioassays. When the plant is a receptor, the variables describing duration of exposure, number of exposures that occur, and intervals between successive exposures are almost co-equal to concentration of pollutant in the prediction of possible or potential effects.

3.3.2. Secondary

Two tests with higher plants were proposed as other candidates for Level 2 screening: seedling growth and seed germination. Several reasons favored the use of these bioassay systems.

(a) Availability: a considerable body of literature and data is available and procedures have been described in "Test Methods for Assessing the Effects of Chemicals on Plants", EPA-68-01-2249, Final Report, Office of Toxic Substances (OTS), 30 June 1975.

(b) Costs. These bioassays are relatively small in scale, require easily acquired facilities, and are relatively inexpensive.

(c) Comparability. Considerable data is available for quality assurance and to determine relative toxicity. Inasmuch as OTS may recommend these tests, a degree of concurrence will be achieved.

(d) Relevance. Firstly, the tests could be developed to accomodate gaseous, liquid, or solid samples and thereby be more compound-oriented (as would be consistent with Level 2 fractionation) and more independent of the phase of the feedstock- or effluent-stream. Secondly, two critical phases in the growth and development of higher plants would be studied. Also, the responses have meaningful end-points. Thirdly, the combination of compound-orientation and the germination of growth of seedlings would be significant in terms of the potential effects of environmental accumulation of materials, especially in the soils.

3.3.3. Tertiary

Some discussion was given to the suitability of other tests, e.g. chromosome breakage in buds and protoplasmic streaming in stamen hairs of Tradescantia; pollen germination, pollen-tube or root-hair growth, and other tests. These were relegated to the area of possibilities to be explored later owing to the use of a more standard test for mutagenicity, difficulty of interpretation, and the more holistic characteristics of the tests above.

4. Decision-Criteria: Level 1

The discussion as to what decision-criteria should be specified for the transition from Level 1 to Level 2 screening in terrestrial systems left this question unanswered and also raised the question of what relevance one level had to another in terrestrial systems. The general consensus appeared to be that: (1) multiple decisions are involved; (2) results for any terrestrial bioassay system cannot be used independently from those of the other bioassays or sampling and chemical analysis; (3) an *ad hoc* decision may be needed for each stream or each chemical based upon its characteristics, once analyses have been made.

In my opinion, the decision-criteria for terrestrial systems cannot be formulated until certain problems are resolved and the roles played by these bioassays are made more explicit in the phased approach , especially in view of the unknown frequencies of false positives and negatives.

4.1. Hierarchy

It would appear that more systematic and extensive exposition of decisions that are involved in the phased approach would be worthwhile. The results of any bioassay, such as the stress ethylene, must answer at least two major questions -- "What rank should be assigned to this stream as a control priority?" "Should Level 2 bioassay be done?" However, there are other decisions that should

be made first, such as, "Should another sample be taken and re-tested at Level 1?" and "Is this a valid result?". The order in which these decisions should be made is probably the inverse of the order in which they are listed. In view of the role played by integrity of sample in the plant-bioassay, the latter two questions would most likely be asked with respect to negative results.

It should be noted that all except the first question can be answered in the metric of yes or no. Therefore, it would probably be better to phrase the first question as a compound, e.g. "Should this stream receive priority, if so at what rank?"

4.2. Contingency

One general conclusion, except for the mutagenesis test, was that decisions for Level 2 are contingent upon the results of chemical screening and other bioassays. With respect to the plant bioassay, it appeared that a highly positive (toxic) response in itself or that slight toxic response and high mammalian toxicity indicated fractionation and Level 2 bioassay were adviseable.

4.3. Validity

It appeared that means for the validation of decisional models or criteria for decisions were not developed to any extent. Nevertheless, two kinds of validity checks are more or less implied in the phased approach with respect to prioritization of streams.

Firstly, the entire battery of bioassays at a level appear to be a check of the validity of the quantitative measure -- degree of hazard (DOH). However, those weighting coefficients (MAC's or TLV's), which enter into the computation of the DOH, may have been derived from the same bioassay systems. Thus the bioassays offer an independent check on the suitability of a linear additive model to derive a measure of DOH mainly with respect to: (1) possible synergism of components of a stream; or, (2) possible effects of one or more unknown but highly toxic components.

Secondly, the utility of the bioassays themselves seems to be compromised by the manifold of different results that may be obtained. If one assumes that twelve bioassays (three each for mammalian, fresh water, salt water, and terrestrial systems) are run with results measured on the scale of no, low, moderate, or high toxicity, then there are 4^{12} possible outcomes. Obviously because of commonalities in the assays, not all outcomes are equiprobable. Nevertheless, it would seem adviseable to drive the biological tests numerically with data on hand (from the numerous, known compounds already screened independently through the bioassay-system) to determine the kinds of events (sets of outcomes) that can occur, the probability of certain events, and their significance with respect to the decisions to validate whatever criteria are chosen.

D.C. McCune
20 November 1978

APPENDIX F

R. Rogers
University of Nevada, Las Vegas

A report submitted to Larry R. Waterland, Leader of the Process Analysis Section of the ACUREX Corporation in Fulfillment of Subcontract RB 82544A.

Part I. General Summary of ACUREX Workshop

In this section of the report I will discuss in a general way the outlined needs IERL has for an EA program and the plant-animal bioassays. Since my expertise is soil microbiology, I will reserve specific comments on the soil bioassays for the next section of the report

I inferred from Ray Merrill's presentation that there is a very urgent need for usable bioassays to compliment existing chemical tests in order to characterize the hazardous of effluent streams. As was explained, rapid, cost effective terrestrial bioassays are required for level I screening of effluent streams with a somewhat more complex and time-consumming assays being used to confirm or refute the findings of level I.

Tables 6 and 7 of document E were presented to show the correlation between chemical and biological testing. It appreared to me that the correlation was not as predictive as would be hoped for. This is especially evident in Table 7 data where DOH's do not always compare with biological response. I assume that these data can be used to emphasize the necessity of a biological screen. On the otherhand, however, the discrepancy might be that either the wrong chemical evaluation index is being used or more important, the bioassays are not responsive to hazardness of the stream. I would suggest that these possibilities be explored.

Other information was given which underscored the difficulty of preserving the integrity of gas samples. Discussion of this subject was raised several times throughout the meeting. Two possible methods of solving this problem were expressed. One was to improve upon the method and containers used for sampling and the other was to push for the use of methods in the bioassay area which could use smaller volumes of gas. It was suggested that the development of miniaturized bioassays systems be persued for gas evaluation studies. Such a system would use less gas hence the problems associated with gas handling would be reduced.

I was personally very pleased that terrestrial bioassays are being considered. There has been a great deal of effort both in industry and government to influence policymakers to only require aquatic assays. Any document written on terrestrial bioassays will certainly bring welcome emphasis to a neglected subject.

The discussion on plant bioassays seemed to be centered on whether or not the stress ethylene plant test was the most acceptable system for level I testing.

During the insuing discussion several plant bioassays were purposed. It was finally decided that given the constraints of cost, sample type, relevance to need, availability of test materials, comparability of data output, and response that the stress ethylene procedure was the logical method to use.

It was also understood that terrestrial plants not aquatic plants would be used. The stress ethylene test appears to lend itself very well to gaseous assays. Apparently there is a fair amount of data which has been obtained from at least two different laboratories who have used the procedure.

The final list of tests for the level I plant bioassays were:

(1) for gas streams - stress ethylene, and foliar injury
(2) for liquid streams - seed germination, and seedling growth
(3) for solids - seedling growth

Some concern was voiced over the media in which the seedlings were going to be grown. For example, if vermiculite is used something should be done to ameliorate its toxic properties.

Level II plant bioassays should consist of more long-range growth, development and reproduction testing. Such testing could include the use of microcosms.

Animal bioassays centered on the use of honey bees, drosophila, and mosquito. The use of these assays seems to be very straight-forward. Much data has been generated with the systems and they lend themselves to the testing of all three phases of streams. These tests can be used for both levels I and II.

Part II. Discussion of Soil Bioassays

The hopes of the soil bioassays was apparently being based on using soil core microcosms for both level I and level II testing. Such testing uses respiration and nutrient export as markers for toxic insult. I am not in favor of using this assay for level I testing because the assay requires too much time for completion (some 8 weeks) to be considered as a rapid screen. Another criticism of the system is that the effect of complex toxic materials has not been determined. For example, if Ca export is being used as an index of perturbation would Ca in the stream being applied to the system cause erroneous results. Also, no data has been generated which will allow for a determination of the applicability of using gaseous and liquid streams. In my opinion this system is still in the developmental stage and can not be considered as an "available" bioassay. This is not to say that the system does not hold promise of being a worthwhile assay. With development, its use as a level II test could be most useful.

Other tests were purposed which would rely on specific microbial functions. Such tests included nitrogen fixation, nitrification, hydrogen oxidation, the degradation of certain key compounds such as starch, cellulose, pectin, and protein, and lastly microbial respiration. Of these methods, endogenous CO_2 production (a measure of respiration), and nitrogen fixation (as measured by C_2H_2 reduction) were chosen.

There are some good reasons why these methods should be used. Both tests are rapid and uncomplicated. A change in CO_2 production reflects an initial change in the metabolism in some or all of the heteratrophic microbiological population. However, it has some problem in that given time unaffected members of the population can cause the CO_2 flow to return to the pretreatment level. On the otherhand, such a deficiency is balanced by using another test which determines

stream affects on a specific group of organisms i.e. dinitrogen fixers. I would certainly urge the concurrent development of tests which use other specific groups of organisms since different toxic materials can affect different microbes in a variety of ways.

Since I have used the hydrogen oxidation assay on different gaseous and solid toxic materials I would suggest that this method be further developed and evaluated. There are several references available on the ease of use, rapidity of the test, ubiquity of oxidizing organisms, and the effect of toxicants on the oxidation process (ref 1 through 7).

Three reference chemicals were suggested for use with the soil bioassays. These were silver compounds (I would suggest $AgNO_3$), sodium azide, and 2-4 dinitrophenyl-ethyleneoxide.

In summary, I feel that the suggested soil bioassay test have promise of providing useful data. However, more time will be required before the two level I tests and the level II soil core microcosm systems can be validated. Since this test will require further data base development I strongly urge that the hydrogen oxidation test also be evaluated at the same time.

Robert D Rogers 11/24/77

ROBERT D. ROGERS, PH.D.
Soil Microbiologist
University of Nevada-Las Vegas

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APPENDIX G

D. Shriner
Oak Ridge National Laboratory

Terrestrial Bioassay Workshop
Key Points of Discussion

David S. Shriner
Research Ecologist
Environmental Sciences Division
Oak Ridge National Laboratory

A. General Summary

Ray Merrill began the workshop by presenting an overview of the phased approach to Environmental Assessment being developed by EPA/IERL, and outlined for us the specific requirements for a bioassay protocol. From my point of view, the most important aspects of this discussion with regard to our task for the remainder of the workshop centered around the need for the level one bioassays to be true screening tests aimed at a preliminary ranking or prioritization of process effluents. Perhaps the most important point from the standpoint of limitations on bioassay systems is the limited sample size likely to be available under current protocol. I feel that sample size should be given some serious thought in relationship to bioassay requirements. Is it conceivable that modification of the sampling protocols to accommodate the bioassay protocols would be more effective and easier in the long run than trying to work with the bioassay systems under limiting conditions of sample size?

Other questions of a general nature which recurred throughout the course of our two-day discussion had to do with sample collection, handling and storage. For liquid and solid samples, these problems are relatively straightforward, and do not seem to me to pose any significant barrier to use in bioassay protocols. Gaseous sample collection, handling, and storage, however, represent what I perceive to be a significant problem which must be dealt with before any widespread use of the gaseous phase testing can be promoted. Of course, the more reactive the species in

the sample, the greater the danger of significant transformations occurring during transport or storage, and it is conceivable that some of these transformations might be missed under routine sampling conditions. For example, consider a sample of tail gas from a sulfur recovery plant. The sample will contain a mixture of sulfur species, probably including H_2S, COS, CS_2, and SO_2. Our on-line monitor will record total S concentration at the time of sample collection, and a second analysis for total S at the time of use of the sample would likely confirm the presence of similar quantities of total S in the sample. However, speciation may have changed dramatically between the two analyses, resulting in a significantly different response of plants to the sample in, for example, the stress ethylene test. Our discussions did not resolve this point to my satisfaction, and I feel it is an area, based on my personal experience with transport of gas samples, that still requires some research effort.

I was interested in the brief discussion of on-site vs. off-site bioassays. My feeling is that while some important advantages might be gained by on-site bioassay testing, by far the most desirable circumstances in virtually all of the bioassay tests - especially from the aspect of minimizing operational problems and maximizing quality control and quality assurance measures, will be to conduct the bioassay tests at a permanent, off-site location.

We discussed a number of attributes of bioassay test systems which are desirable and/or mandatory (cost, sample type, relevance, availability, comparability, and response).

Of the above, cost, while important in the overall picture, can probably be eliminated as a factor in bioassay test selection since most of the tests meeting the remainder of the criteria will likely fall within a relatively narrow range of costs anyway.

Relevance is an important point in test selection. It is very important that the test selection be geared to likely pathways of exposure in natural systems (i.e., if contamination of soils is suspected, a test measuring foliar injury of vegetation would probably be inappropriate - even though foliar injury might result in some cases. Obviously a test known to be consistently sensitive to stress on root systems might be expected to be more sensitive, and we are left with seed germination, seedling growth, and stress ethylene tests as more likely candidates.

We reviewed a number (10 by my count) of potential phytotoxicity bioassays and came to a general agreement that stress ethylene and seed germination tests are the two best of the currently available options. A large data base exists for seed germination testing, and a growing data base exists for the stress ethylene test. I will make additional comments in the area of phytotoxicity testing in Part B, specific comments in my area of expertise.

The discussion on soil bioassays included, again, a number of available tests. Perhaps the first accomplishment of this discussion session was to thoroughly discuss the merits of the proposed soil-core microcosm test. I felt satisfied that our discussion of the method and its potential strengths and weaknesses left little room for doubt that this method is unsuitable - at least at the present time - as a level one assessment tool. I see a couple of major problems which were fundamental in the arrival at this conclusion: 1) Most of the proposed measurement parameters, involving nutrient efflux (Ca^{++}, $PO_4^{\equiv}$, $SO_4^{=}$, NH_4-N, NO_3-N) are potential constituents in effluent streams. Furthermore, in many energy-related process and waste streams, Ca^{++} and/or $SO_4^{=}$ are present in relatively significant quantities. In such cases, measurement of calcium efflux from a soil core microcosm would present more problems in interpretation than I would consider acceptable for a level I bioassay; and 2) My impression from our discussion and the

comments of those more familiar with this test than I, is that there is a great deal of ambiguity associated with interpretation of the results. Furthermore, there are not sufficient data yet available to make any real assessment of the frequency of false positives or false negatives. I feel that it is a key point that not only must these bioassays be rapid, inexpensive and reproducible, but they must lend themselves to very clear-cut, straightforward interpretation of results. A "yes" or "no" answer is required, and every effort should be made to minimize the frequency of "maybe" results, since each such questionable response must be treated as a positive.

There appeared to be general agreement that endogenous soil respiration measurements are one indication of soil microbial activity for which adequate data exist to establish this type of test as reasonably replicable. A 30-day test was recommended to permit stabilization of the system after manipulation.

Endogenous respiration measurement does not appear to be adequate to stand alone as a soil bioassay. It was therefore suggested that a second bioassay be included in the protocol, with nitrogen fixation and nitrification assays, because of their broad use in agricultural work - an established data base - and their relative sensitivity, being recommended. Of the two, consensus was for the acetylene reduction assay for nitrogenase activity, a 24-hour test. Each of these tests were thought to be applicable to liquid, solid, or gas samples.

In addition, it was agreed that the hydrogen oxidation test discussed by Rogers should be given development priority.

All of the soil tests selected necessarily call for the evaluation of basically site-specific soils and soil microbial populations. For the purposes of IERL this may not constitute a significant problem if the tests are to be conducted in-house, at a limited number of sites. However, should

others wish to reproduce the tests, it may well be worth specifying that soil chemical and physical parameters be as fully characterized as possible. Some degree of standardization of loam, sand, and clay fractions, exchange capacity, and organic matter content could help. Textural variations alone could influence moisture status at micro-sites of biological activity within the soil matrix, and contribute to unacceptable variability in response data.

We discussed synthetic soil preparations which might be standardized, but there was strong feeling that this was an approach which had been unsuccessfully explored in the past, and was probably not worth additional effort. It would seem to me, however, that soil microbial populations might be able to be standardized. This could conceivably be accomplished by soil sterilization - perhaps by gamma irradiation, and a subsequent standard reinoculation of soil microflora and fauna. Such standardization might make replicability, reproducibility, and data interpretation easier.

Animal tests were discussed briefly. Because of the large volumes of data existing on both honeybee and drosophila as test organisms, it was generally agreed after discussion, that acute oral LC_{50} tests for liquid and solid samples would be appropriate, and that the same organisms could also be used in exposure to gaseous samples as well.

A discussion occurred on what should be the decision criteria for specifying Level 2 tests on the basis of Level 1 results. I was favorably impressed with the use of the Maximum Applicable Dose concept where it is appropriate. Once the Level one response has been categorized as either high or low, it was suggested that two different types of Level two assessments might be appropriate depending upon the type of Level 1 response observed. If level one testing established a high toxicity level in a particular sample, fractionation of the sample (sample size permitting) and

re-running of the Level one tests with each of the fractions would be an appropriate Level two response. If, however, a given sample tested high in only one test, or moderate in several tests, the appropriate Level two response for those tests lower in toxicity might be to rerun the test with additional species to confirm the potential hazard before going to the expense of fractionation.

Using the above scheme, stress ethylene and foliar injury would be utilized as both Level one and Level two tests as would seed germination. Based on Level one, or other Level two data, full life cycle studies were also regarded as a Level two test for plants.

Endogenous soil respiration and acetylene reduction were Level one soil tests, with soil core microcosm and specific substrate respiration suggested as Level two tests for soils.

Animal testing should employ the acute LC_{50} tests with honey bee and Drosophila as both Level one and Level two test, with complete life cycle, bioaccumulation, and behavioral tests being included as potential Level two tests if appropriate.

There was discussion on the need for inclusion of blanks, reference samples, and positive controls in the testing scheme for all tests. The need for these steps cannot be overemphasized in my opinion. We discussed on numerous occasions the variability and frequency of false hits with each of these tests. Any meaningful interpretation of data from such a testing protocol will have to utilize positive controls and appropriate reference chemicals to establish standards for comparison. I would recommend, in the case of phytotoxicity in seed germination and seedling growth tests, the adoption of the 10 chemicals currently being used by Tingey in the round-robin experiment as standard reference chemicals. For soils, 2,4-D, silver, cadmium, and sodium azide were suggested as appropriate reference chemicals. And for animals, parathion and Monosodium metharsenate (MSMA) were suggested.

For standard reference gases, ozone, clorine, hydrogen chloride, and ethylene oxide were suggested as references. I would comment here that chlorine gas and HCl gas behave somewhat similarly in terms of their effects on plants, and would, in my opinion, represent an unnecessary duplication.

I would like to now offer comments on some of the specific questions (on page 4 of our agenda handout from the workshop) which have not been addressed elsewhere:

- Effects of sample integrity - although we discussed this problem on several occasions, I don't feel that it was ever resolved in a totally satisfactory manner. The samples of most critical concern from the standpoint of sample integrity will be, in my opinion, the gaseous samples. My opinion, from frequent discussions with chemical engineers on our staff about this exact problem, is that the solution is an engineering problem. Given the resources, it is a solvable problem, albeit a costly one.

- Effects of sample treatment procedures - also a real problem. I would certainly opt for sample treatment procedures which would optimize the utility of the samples for bioassay purposes. The introduction and subsequent removal of toxic solvents from a sample could seriously alter the sample's integrity.

- Can Level one tests accommodate samples from Level 1 sampling? Given the choice, I would certainly prefer to see separate samples collected for bioassay purposes. One of the biggest reasons for this is the limitation of sample size. A SASS train sample is not large enough in most cases to permit Level I and II testing from the same sample, especially if fractionation were required.

- Should solids be tested directly...? Both solids and leachates, or extracts of the solids should be tested, in my opinion. A toxic response to a solid could be attributed to a high concentration of a heavy metal, by analysis. However, the heavy metal might not have been in a soluble form, and the toxic response could have actually been due to highly soluble sulfate, for example.

- Should samples be concentrated? If an octanol/water partition coefficient were to suggest potential for bioaccumulation, and especially if mutagenic or teratogenic activity were suspected, concentration would be called for. For simple toxicity tests, however, we can expect chemicals in the environment to generally undergo dilution during environmental transport, making concentration of the samples a probably unnecessary step.

- Does Level 2 testing require new (fresh) samples? It is probably unrealistic to expect samples to maintain their integrity from collection through to the completed assessment of the Level 1 test in a state acceptable for Level II testing. However, if fresh samples were to be collected for Level II testing, I would recommend simultaneous repeats of the Level I tests on the fresh sample. The costs would be minimal, and since you have already flagged the material as potentially problematic, it would seem to me you couldn't afford not to rerun the Level I tests concurrent with the Level II tests.

B. Phytotoxicity Assays

I wish to make only a few additional comments to the ones I have already made under "general comments".

First of all, I would encourage IERL to be aware of the potential impact of their planning and protocol development exercise. Even though the tests are presently planned for in-house use at EPA labs, because of their applicability to current requirements of enforcement arms of EPA under TSCA and RCRA, these tests could easily find themselves being used by hundreds of private commercial testing labs with varying standards of quality assurance. For this reason, every possible effort should be made to make the protocols as clear-cut and unambiguous as possible.

Along this line, perhaps the most critical step in getting reliable, reproducible results from seedling growth and stress-ethylene testing will be plant culture conditions. I would strongly urge reliance upon the

materials developed for this purpose by Ted Tibbitts and his subcommittee associates of the American Horticultural Society.

Three recent current versions of the seed germination test have been tested by various labs. I have no experience with the Franklin Institute version currently favored by TSCA, but can speak directly to the versions being used at Corvallis and Oak Ridge. My opinion is that there are no significant differences in principle between the two versions. They should be cross-calibrated with one another, but I would not anticipate any significant differences in response.

One final comment - I am personally troubled by the proposed static exposure conditions for gaseous sample testing of plant response. I feel that it is extremely difficult to make valid assessment of dose-response under static exposure conditions, and I would urge at least further consideration of flow-through exposure conditions for the implementation of stress-ethylene, foliar injury, and seedling growth tests.

APPENDIX H

T. Tibbitts
University of Wisconsin

Report on Workshop

Terrestrial Ecology Bioassay Protocol
for Environmental Assessments Program

T. W. Tibbitts

University of Wisconsin - Madison

Summary of Meeting

A. Level 1 Protocol

The participants at the meeting concurred in the following outline of protocol for the Level 1 bioassays.

Plants	Soil Microorganisms	Animal
Rate of ethylene production	Respiration of soil population	Honey bee survival
Foliar injury	Acetylene reduction of soil	Drosophila survival
Seed Germination percentage		
Seedling growth		

These protocol were accepted because all have been reasonably well defined and have been utilized either in other bioassay programs or evaluated in more than one laboratory as useful bioassays for the Environmental Assessments Program.

It was apparent that the group were more comfortable with recommending the animal tests because of the large amount of use and standardization that has been developed for these assays in pesticide evaluation. (This should be documented, however)

There were greater reservations for recommending the plant and soil microorganism tests because none of the assays have been adequately standardized for bioassay use and/or evaluated for use with mixed 'stream' effluents.

A summarization of the group response to each of these bioassays is as follows:

Plants

Rate of ethylene production - This should be an effective bioassay for it provides a non-specific response of plants that monitors many different types of stress reactions in the plant. It is known to occur with water stress, pressure, chemical toxicity and any tissue injury. It also monitors a very relevant response of plants,ie non-normal stress or injury to the tissues. The test has been duplicated successfully in two different laboratories.

Foliar Injury

Has been utilized effectively in many types of pesticide testing and could be utilized in conjunction with the ethylene production assay to reduce greatly the cost of this assay. The fact that the Office of Toxic Substances was to include this bioassay in their recommended procedures encouraged its inclusion in this protocol.

Seedling Germination Percentage

Procedure for this have been described in the EPA 560/5-75-008 report of Test Methods for Assessing the Effects of Chemicals on Plants. This could provide a test requiring a minimum amount of space and minimum amount of environmental control. It was felt that this could be utilized with gaseous toxicants even though germination would have to be on a moist substrate.

Seedling Growth

The group encouraged this test to provide evaluation of morphological development of plant systems. It also could be conducted in a small system and requires a minimum of environmental control for the first level of assaying. It could be conducted without light. It was indicated that this test could be combined with the seedling germination test to minimize cost.

Soil Microorganisms

Respiration of the soil community - The group supported this bioassay because it was a very basic response for providing energy in all living systems and for the decomposition of organic matter. It also was a rather non-specific response that might be altered by toxicity to one of several different metabolic systems within organisms. It was obvious, though, that the system might be overly sensitive and respond to nearly any alteration of the physical or chemical soil system even though the alteration was not a distinct chemical toxicity. The group also expressed the concern for false negatives, for respiration might be stimulated by the addition of organic substrate in the stream effluents.

Acetylene reduction of Soils

The group supported this assay because of its basic relevance to nitrogen fertilization for plants and because there has been considerable development of this procedure for the extensive assay work in soil nitrification.

Honey bee survival

The group supported this assay because of the very high value that society places upon honey bees and because careful testing procedures have been developed for evaluation of pesticide toxicity. There was some concern expressed for the problems of maintaining active colonies at all times, but this did not seem to be a serious limitation. Insect tests are useful animal tests because of the large populations that can be evaluated with very small samples. They are particularly useful, thus, for gas samples.

Drosophila survival

The group supported this bioassay because of the very detailed research data available on drosophila and its response to different types of toxicants. There

also is good information for genetic response evaluations. The very small size of this organism also is of considerate advantage. The lack of significant relevance to life on this planet is the biggest disadvantage of this test.

B. Level 2 Protocol Recommendations

Plants	Soil	Animals
Full Life Cycle	Soil core microcosim tests	Full Life Cycle
	Substrate Respiration	Behavioral

There was no attempt to carefully detail Level 2 studies but recommendations were made to provide a basis for encouraging investigation of acceptable standardized procedures for each.

Plant Full Life Cycle

It was proposed that there was a need to follow plants from seed to seed to determine toxicity to plant responses not studied in the seedling plants or foliar injury studies. Responses that should be studied include reproductive initiation, flowering phase initiation, sexual tissue development, and fertilization. It could also permit study of genetic changes. Arapadopsis was suggested as a useful plant species for it has been grown from seed to seed in test tubes in a minimum of space.

Soil Core Microcosm

This test was proposed for Level 2 testing instead of Level 1 because no effective means of standardizing the soil to be used in each test was able to be recommended and because the response tests involve determinations for chemicals that may often be present in the stream placed on the microcosm. Thus, the results obtained may not be definitive enough to establish toxicity effects.

Substrate Respiration

Recommended for Level 2 because each substrate utilized had a rather restricted relevance to the functioning of the whole organism, and therefore, a large number of substrates, involving excessive expense, would be required to get meaningful results.

Animal Full Life Cycle

No particular animal studies were proposed although bee, drosophila, flies and mosquitos should be investigated because standardized procedures are available for all.

Behavioral

No particular animal studies were proposed, however, comments indicated support for using bees for these studies.

The following proposed studies were not included in this list for the following reasons:

Hydrogen oxidation

This soil microorganism test appears to have usefulness for bioassays but required additional evaluation and standardization to make useful. It is a very specific test of unknown relevancy but would likely have little interference from materials in the stream.

Plant tests

Plant responses including pea epicotyl growth, bean hypocotyl opening, pea tendril, tomato epinasty, turgor swelling, pollen growth, and cucumber leaf enlargement were not recommended because these tests either were very specific response test, i.e. hormone response, or were too difficult to standardize for this bioassay testing.

Tradescantia stamenal hair alterations.

This bioassay procedure was not encouraged because of the complexity of maintaining flowering plants in an available test form over the entire year.

Soil nitrification

This test was excluded because it was a rather specific test involving nitrogen availability to plants and thus, closely paralleled the actylene reduction test that was included. It might be considered as an additional Phase II test.

Mosquito and house fly survival

This test was excluded because it closely paralleled the drosophila test and thus, was an unnecessary duplication of effort.

The following additional recommendations were made by the committee:

Level 2 testing would be undertaken when Level 1 bioassays indicated toxicity however, there would have to be some judgement involved in each specific case to determine what Level 2 bioassays should be undertaken.

C. Types of Level 2 Testing Recommended.

1. Fractionation of streams for the purpose of identifying the specific toxicants in the mixtures of Level 1 testing.

2. Longer term testing to establish chronic effects of toxicants.

3. Test for toxic effects on all stages of plant growth, development, and reproduction.

4. Determine if toxicity occurs in several different species.

D. Samples for Inclusion in Bioassay.

1. Sample at least three concentrations of stream effluent.

2. Control sample without stream effluent.

3. Control sample with a reference toxicant.

4. Control sample with a blind toxicant.

E. Bioassays are required for the following reasons:

1. Confirm the expected degree of toxicity for chemicals known to be in the stream.

2. Establish toxicity from unrecognized chemicals in the stream.

3. Determine unrecognized toxicities from interacting levels of 2 or more chemicals in the stream.

F. Specific Comments in My Area of Expertise.

1. Level 1 Plant Experiments

a. There is a need of carefully detailing the environmental conditions and growing procedures for all plant experiments. This should include the following factors: (See attachment for examples of standardized growing conditions.)

Seed

Seed supplier and storage conditions

Regular germination tests to insure seed vitality

Selection of a self-pollinated plant or hybrid to reduce genetic variability

Cultural Procedures

Media composition

Compaction of media

Seed sowing depth

Nutrition of media

Watering procedures

Rotation of plants in chamber